AN EXAMINATION OF THE BIOACTIVE LIPIDS INVOLVED IN SKIN CELL INFLAMMATION AND IN RESPONSE TO ULTRAVIOLET RADIATION

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An examination of the bioactive lipids involved in skin cell inflammation and in response to ultraviolet radiation

Effect of n-3 polyunsaturated fatty acid supplementation on red blood cell and human dermal fatty acid and production of eicosanoids by HaCaT keratinocytes and 46BR.1N fibroblasts following exposure to UVR.

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Abstract

Ultraviolet radiation (UVR) in solar light is important for skin biology. It is involved in the development acute and chronic skin inflammation, aging and cancer, causing erythema, tanning and local or systemic immunosuppression. Omega-3 polyunsaturated fatty acids (n-3 PUFA) are considered anti-inflammatory and could reduce the damage caused by overexposure to UVR. Although, n-3 PUFA have been considered as photoprotective agents, their exact mechanisms of action is not completely understood.

The aim of the work is to determine the effect of UVR and the n-3 PUFA eicosapentaenoic acid (EPA), or docosahexaenoic acid (DHA) on human skin cells (in vitro study), specifically on: cell viability, apoptosis and their metabolism through the cyclooxygenase and lipoxygenase pathways. Also, to study the cellular incorporation and effect of n-3 PUFA on the fatty acid profile of skin cells. A clinical study was undertaken to assess the incorporation of n-3 PUFA supplements in human skin.

A clinical study was performed in 40 healthy women (active group) supplemented with 4g/day of EPA (70%) and DHA (10%) and 40 healthy women (placebo group) supplemented with 4g/day of glyceryl tricoprylate coprate (GTCC). After 3 months, both blood samples and skin punch biopsies were collected and analysed for fatty acids by gas chromatography (GC). HaCaT keratinocytes and 46BR.1N fibroblasts were cultured and treated with 10 and 50μM of either EPA, or DHA or oleic acid (OA) for 72h and exposed to 15 and 50 mJ/cm^2. Cell viability was measured by the MTT assay and cell apoptosis by a colorimetric method, at 24h post UVR. Cells and culture media were analysed by GC and liquid chromatography tandem mass spectrometry (LC/ESI-MS/MS) to assess cellular fatty acids and production of eicosanoids.

The clinical a study showed that in RBC saturated fatty acids (SFA) (44.27±7.43%) were the main fatty acid group followed by n-6 PUFA (29.61±5.53%). While in dermal tissue monounsaturated fatty acids (MUFA) (58.90±9.80%) was the main fatty acid group followed by SFA (27.06±6.78%). A significant increase in EPA, DHA and docosapentaenoic acid (DPA) was observed in RBC but only EPA was significantly increased in the dermis post n-3 PUFA supplementation. .

The viability of HaCaT keratinocytes and 46BR.1N fibroblasts decreased post UVR and this was further reduced post PUFA treatment. Cell apoptosis increased when cells were exposed to UVR and further increased when cells were treated with EPA and DHA. .

In HaCaT keratinocytes MUFA (54.22±8.82%) was the main fatty acid group followed by FAS (37.11±.9.16%), while SFA (51.94±8.68%) was the main group followed by MUFA (27.07±4.79) in 46BR.1N. Treated both cells with EPA and DHA showed significant increased in cellular EPA, DPA and DHA. 46BR.1N fibroblasts produced higher levels of prostaglandins (PG) compared to HaCaT keratinocytes: PGE_2 and PGD_2 were the main PG in both HaCaT (7.96±3.18 and 1.48±1.19 pg/million cell; respectively) and 46BR.1N with (44.2±23.00 and 17.1±9.71 pg/million cell; respectively). Significant increase in PGE_1 and PGE_2 occurred when cells were exposed to 15mJ/cm^2 UVR. Treatment with n-3 PUFA decreased the level of PGE_1 and PGE_2, and increase production PGE_3 at the baseline and post UVR. Both cell lines produced hydroxy fatty acids and the concentration of these mediators was higher in 46BR.1N than HaCaT. The concentrations of these mediators were significant increased post UVR: treatment with n-3 PUFA decreased the level of HODE and HETE, and increase production of HEPE and HDHA at baseline and post UVR.

Overall, n-3PUFA treatment led to increases in the content of EPA and DHA on RBC, dermal tissue and human skin cell lines. EPA and DHA in skin cell lines appear to offer protection by increasing cellular apoptosis, decreasing inflammatory mediators specifically PGE_2 and 12-HETE, and increasing anti-inflammatory mediators such as PGE_3, 15-HEPE and 17-HDHA.

Published papers

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Suzanne M Pilkington, Lesley E Rhodes, Naser MI Al-Aasswad, Karen A Massey and Anna Nicolaou. 2013. Impact of EPA ingestion on COX- and LOX-mediated eicosanoid synthesis in skin with and without a proinflammatory UVR challenge. Report of a randomised controlled study in humans. *Molecular Nutrition and Food Research.2013,00,1-11*

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15-epiLXA4	Aspirin-triggered lipoxin
15-PGDH	15 hydroxyprostaglandin dehydrogenase
46BR.1N	Human fibroblast cell line
5(S)-HETE	5(s)-hydroxyeicosatetraenic acid
AA	Arachidonic acid
AD	Atopic dermatitis
ALA	α-Linolenic acid
BF_3	14% boron trifloride in methanol
BHT	2,6-di-tert-butyl-4-methylphenol
BLT1	leukotriene B4 receptor
cAMP	cyclic adenosine monophosphate
CMKLR1	Chemokine-like receptor 1
COX	Cyclooxygenases
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CPD	Cyclobutane pyrimidine dimmers
cPGES	Cytosolic PGES
DGLA	Dihomo γ linolenic acid
DHET	Dihydroxyeicosatrienoic acid
DHETs	Dihydroxyeicosatrienoic acids
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECACC	European Collection of Cell Culture

EDP	Epoxy docosapentaenoic acid
EDTA	Ethylenediaminetetraacetic acid
EET	Epoxy-eicosatrienoic acid
EPA	Eicosapentaenoic acid
FAEE	Fatty acid ethyl ester
FAME	Fatty acid methyl ester
FBS	Fetal bovine serum
FCS	Fetal calf serum heat inactive
FLAP	5-lipoxygenase activating protein
GLA	γ linolenic
GPCR	G-protein-coupled receptors
GTCC	Glyceryl tricoprylate coprate
H_2O_2	Hydrogen peroxide
HaCaT	Human keratinocyte cell line
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric acid
HETE	Hydroxy eicostetraeneic acid
HETrE	Hydroxyeicos-atrienoicacids
HPDHA	Hydroperoxy docosahexaenoic acid
HPEPE	Hydroperoxy eicosapentaenoic acid
HPETE	Hydroperoxy eicosatetraenoic acid
H-PGDS	Haematopoietic - PGDS
HODEs	Hydroxyoctadecadienoicacids
iPLA$_2$	Independent cytosolic Ca^{2+} phospholipase A_2
IsoPs	Isoprostanes

LA	Linolenic acid
LB	Lipid-enriched lamellar bodies
LOX	Lipoxygenase
L-PGDS	Lipocaline type PGDS
LT	Leukotriene
LTA4	Leukotriene A4
LTB4	Leukotriene B4
LTC4	Leukotriene C4
LTD4	Leukotriene D4
LXA_4	Lipoxin A_4
LXB_4	Lipoxin B_4
MAR	Maresins
MED	Minimal erythema dose
MEME	Minimum Essential Medium Eagle
mPGES	Membrane-bound PGES
MRM	Multiple reaction monitoring
MUFA	Monounsaturated fatty acid
n -3 PUFA	Omega -3 polyunsaturated fatty acid
n -6 PUFA	Omega 6 polyunsaturated fatty acid
NaBr	Sodium bromide
NHAK	Normal human adult keratinocytes
NO	Nitric oxide
OA	Oleic acid
PAF	platelet activating factor
PBS	Phosphate buffered saline

PDs	protectins
PG	Prostaglandin
PGD11-KR	PGD 11-ketoreductase
PGDS	Prostaglandin D synthases
PGE9-KR	PGE 9-ketoreductase
PGES	Prostaglandin E synthase
PGFS	prostaglandin F synthase
PGG_2	Prostaglandin G_2
PGH 9,11ER	PGH 9-,11-endoperoxide reductase
PGH_2	Prostaglandin H_2
PGT	prostaglandin transporter
PLC	Pityriasis lichenoides chronica
PL	Phospholipase
PLA_2	Phospholipases A_2
PUFA	Polyunsaturated fatty acid
RBC	Red blood cells
RCT	Randomized controlled trials
ROS	Reactive oxygen species
RvD	D-series resolvins
RvE	E- resolvins series
SB	Stratum basale
SC	Stratum corneum
SFT	Saturated fatty acid
SG	Stratum granulosum
SPE	Solid phase extraction

$sPLA_2$	Secretary phospholipases A2
SS	Stratum spinosum
TEWL	Transepidermal water loss
TNF-α	Tumor necrosis factor alpha
TXAS	Thromboxane A synthase
UCA	Urocanic acid
UVR	Ultraviolet radiation

Chapter 1. Introduction

1.1. Skin

The skin is a complex multilayered organ with several specialized appendages (Freinkel and Woodley, 2001). The skin is identified as the largest and one of the most important organs in the body (Bos, 2005). It is about 1.8 m^2 in surface area, 1.2 mm in average thickness, and can weigh up to 4.7kg with blood and 4.2 kg without blood. The thickness varies and it can be extremely thin in some areas of the body, such as the eyelids and postauricular area. Overall skin constitutes 6% of body weight (Goldsmith, 1990). Skin has important roles and functions in the human body, due to the variety and unique combination of cells of which it is made. The skin protects the body from external environmental influences, such as chemical, physical and bacterial damage (Madison, 2003) and harmful rays including ultraviolet radiation (UVR) (Creidi et al., 1994) (Figure1.1).

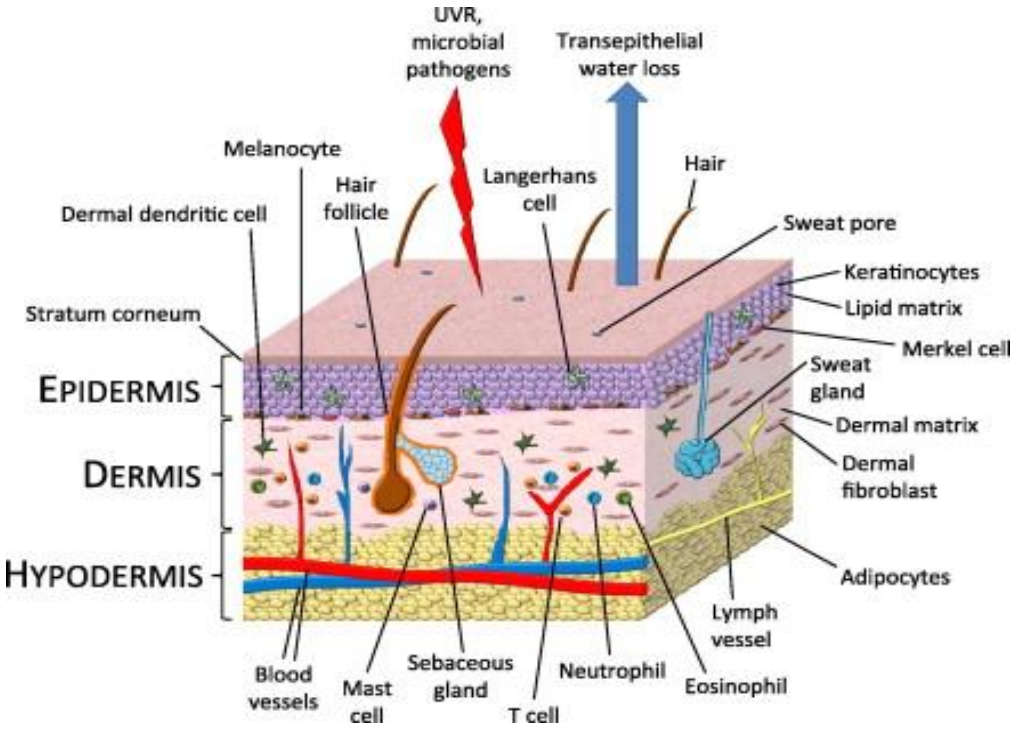


Figure.1.1. Schematic of the structure of human skin (Taken from Kendall and Nicolaou, 2013).

The skin consists of three layers: the epidermis or outer layer, the dermis or middle layer and the subcutaneous tissue or bottom layer (Figure 1.1). Each layer comprises specialised cells, and each has its own role and function.

1.1.1. Epidermis

The epidermis is the external layer of the skin and acts as a physical barrier between the body and the environment. The thickness of the epidermis is about 0.8 – 1.4 mm (Gawkrodger, 2008). The epidermis does not contain blood vessels; therefore it is nourished from the dermis by diffusion. Four types of cells are important for the function of the epidermis: keratinocytes, melanocytes, Langerhans cells and Markel cells. The epidermis is divided into four layers: stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG) and stratum corneum (SC) (Figure 1.2).

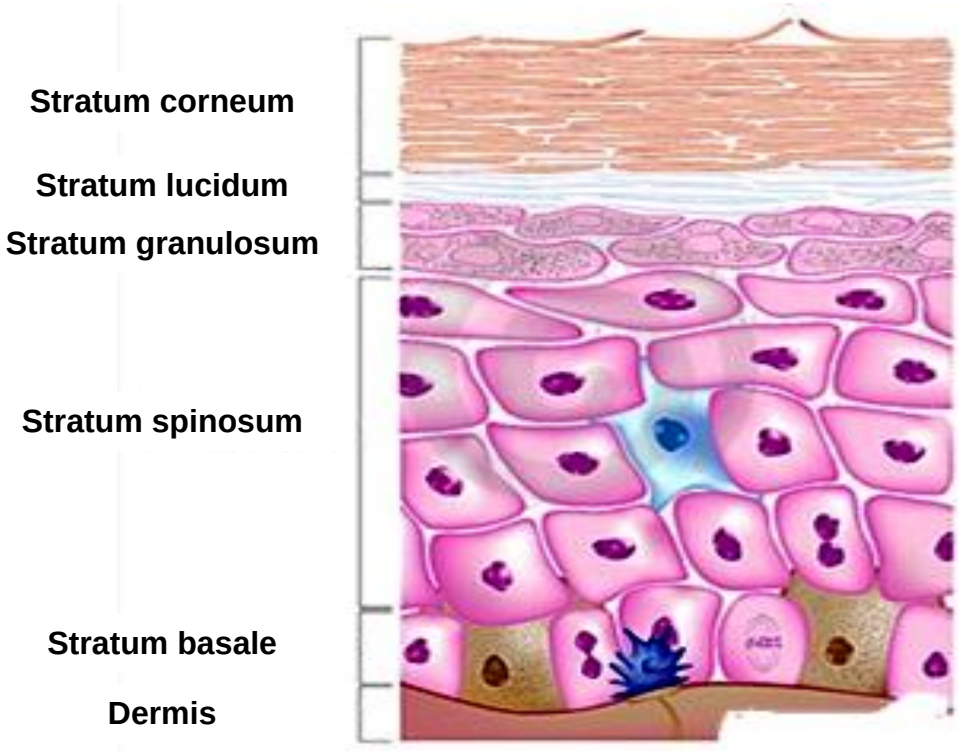


Figure.1.2. Schematic representation of human epidermis (Taken from ww.biomedexperts.com).

1.1.1.1. The layers of the epidermis

1.1.1.1.1. The stratum basale layer

The stratum basale layer (Figure1.2) is a germinative layer that is generally described as single layer of columnar basal cells (Graham-Brown and Bourke, 2007). Every basal cell divides by mitosis every four days to generate new basal cells. The new basal cells constantly push older cells up to the skin surface. The stratum basale cells are attached to the basement membrane by hemidesmosomes (Heyden et al., 1992).

1.1.1.1.2. The stratum spinosum layer

The stratum spinosum layer (Figure 1.2) is directly above the basal layer. In this layer, keratinocytes become polyhedral after they lose their columnar shape, and their ability to produce the amount and types of keratin is also increased (Mottaz and Zelickson, 1975). Lipid-enriched lamellar bodies (LB) (keratenosomes, membrane-coating granules) appear first in this layer. The cells begin to become flat and elongated in the upper layers of the stratum spinosum, and migrate to the stratum granulosum.

1.1.1.1.3. The stratum granulosum layer

The stratum granulosum (Figure 1.2) is above the stratum spinosum layer. Granule cells can be recognised by their basophilic keratohyalin granules and lamellar bodies which are biochemically composed of lipids. The lipid content of lamellar bodies is secreted into the intercellular spaces of the deeper stratum corneum (Freinkel and Woodley, 2001). Keratinocytes lose the ability to differentiate and as a result, cornified cells appear (Candi et al., 2005, Ishida-Yamamoto and Iizuka, 1998).

1.1.1.1.4. The stratum corneum layer

The outer layer of the epidermis is the stratum corneum (Figure1.2). It plays an important role in maintaining life as the barrier of the skin. Moreover it is involved in the regulation of transepidermal water loss. The stratum corneum contains lipid secreted by lamellar bodies. The corneocytes become flat and not nucleated as a result of the final step of the differentiation of keratinocyte (Proksch et al., 2008). Although corneocytes are not alive, they are still biochemically active and are linked and connected together by intracellular lipids (Tobin, 2006, Marks et al., 2006). There is a complex mixture of lipid and proteins between the corneocytes. Keratinocyte secreted enzymes metabolise and break down the intracellular lipids to produce a lipid mixture that contains fatty acids (15%), cholesterol (25%) and ceramides (50%) of the total lipid mass. The lipid mixture is important for the skin's epidermal barrier (Holden et al., 2002).

1.1.1.2. Epidermal cells

1.1.1.2.1. Epidermal keratinocytes

Keratinocytes are the major type of cell in the epidermis. They constitute approximately 95% of all cells found in the epidermis (Rook and Burns, 2004, Lippens et al., 2009). They form several layers from the epidermal base membrane to the skin surface. Keratinocytes are responsible for the production and maintenance of the stratum corneum layer. Furthermore, they participate in immunological and inflammatory processes (Forslind et al., 2004).

Keratinocytes exist in the stratum basale and then migrate from the basal layer to reach the skin surface. As they migrate, they produce different types of

keratins, which are insoluble proteins that help to protect the skin. Keratins type 5 and type 14 are produced by the keratinocytes in the stratum basale layer of the epidermis (Heyden et al., 1992). In the stratum spinosum, the ability of keratinocytes to produce keratins is increased as well as their ability to produce different types of keratins. At the terminal stage of keratinocyte differentiation keratins type 1 and type 10 are the main keratins produced (Heyden et al., 1992). Once the keratinocytes reach the granular epidermal layer, keratohyalin granules and filaggrins appear and the cells become corneocytes. The main types of keratins secreted at this stage are type 6 and type 16.

1.1.1.2.2. Melanocytes

During early fetal development, melanocytes are derived and migrate into the skin from the neural crest (Erickson and Goins, 1995). They are located in the stratum basal layer of skin. They comprise 1-2% of the epidermal cell population and constitute about 5-10 % of the cell population in the basal layer. The main function of melanocytes is to synthesise melanin (Gawkrodger, 2008). Two main types of melanin are produced by melanocytes the red/yellow pheomelanin and the black/brown eumelanin. The synthesis of melanin takes place in the melanosomes. Once melanin is produced the melanosomes are transferred into the neighbouring keratinocytes (Kobayashi et al., 1998). The number and size of melanosomes is important in determining skin pigmentation. They are larger in black skin than in white skin. Each mature melanocyte can make contact with about 30–40 keratinocytes to make the epidermal–melanin unit (De Luca et al., 1988). Ultraviolet irradiation is the main factor that stimulates melanin production (Graham-Brown and Burns, 2007, Gawkrodger, 2008). Melanin protects cells from UVR damage by a) absorbing electro-

magnetic radiation of this wavelength and b) acting as a scavenger of free-radicals which are produced by UVR (Prota, 1997). Melanocytes are also able to produce a wide range of signalling molecules, such as melanocortin peptides (Wakamatsu et al., 1997), cytokines (Förster E et al., 1991), serotonin (Johansson et al. 1998), catecholamines (Iyengar B and Misra RS 1987) and eicosanoids (Masoodi et al., 2010, Gledhill et al., 2010).

1.1.1.2.3. Langerhans cells

Langerhans cells are generally found in the epidermis and in hair follicles within the outer root sheet (Baumann et al., 2003). They are epidermal dendritic cells and Birbeck granules are characteristic of them (Musso et al., 2008). Langerhans cells were identified by Langerhans in 1868 (Langerhans, 1885). As they are not linked to other cells by desmosomes, they can move and migrate from the skin to the immune system (lymph nodes). It is thought that they are able to internalise and process foreign antigens and transport them to the skin lymph nodes where cutaneous immune responses are initiated (Cumberbatch et al., 2003). Langerhans cells migration and maturation is mainly controlled by cytokines that are secreted by the cells themselves end those secreted by surrounding keratinocytes (Griffiths et al., 2005). Also, in response to UVR, LCs migrate to the lymph nodes (Dandie et al., 2001, Allan et al., 2006).

1.1.1.2.4. Merkel cells

Merkel cells were first described by Merkel FS in 1875; these large cells are found in the basal layer of the epidermis (Sidhu et al., 2005). The number of Markel cells is low compared to keratinocytes. Merkel cells are associated with

nerve terminals, forming a Merkel cell-neuron complex, which acts as a mechanoreceptor (Baumann et al., 2003).

1.1.2. Dermis

The dermis is found directly under the epidermis (Figure1.1). It varies in thickness from 0.6 mm in eyelids to 3 mm in the back of the palms and the soles of the feet (Gawkrodger, 2008). The dermis is composed of two layers. The upper papillary layer contains reticulin fibres, elastin and collagen. This layer is adjoined to the epidermis (Washington et al., 2001). The lower reticular layer has fewer reticulin fibres, and an elastin and collagen network that provides structural support. Like any connective tissue, the dermis has many types of cells: fibroblasts which synthesise collagen, elastin and, ground substance, mast cells or mastocytes (which are placed near the blood vessels) and histocytes which are a kind of macrophage. In contrast to the epidermis, sensitive nerve endings and small blood vessels are found in the dermal layer (Starr, 2008).

1.1.2.1. Dermal fibroblasts

Fibroblasts are one of the most important cells in the skin (Sorrell and Caplan, 2004). They synthesise all the components of the protein matrix: collagen, elastin and ground substance. They also, produce enzymes such as collagenases and gelatinases, which are responsible for degradation and remodelling of the collagen matrix (Soter and Baden, 1984). Collagen makes up 90% of the total dermal protein and about 70-80% of the skin's total dry weight (Hunter, 2002). There are at least 25 types of collagen and about half of them are present in the skin. Collagen type I (85–90%) is the most abundant followed by collagen type III (8–11%) and collagen type V (2–4%) (Uitto, 1989).

Elastin is the second protein produced by fibroblasts. It constitutes about 2% of the skin's total dry weight and most of it is located in the dermis (Rosenbloom et al., 1993).

1.1.2.2. Mast cells

Mast cells are considered secretory cells that are found in all dermal regions but are most commonly present in the upper dermis (Soter and Baden, 1984). Mast cells have an important role in inflammatory and allergic reactions, and they secrete bioactive mediators such as histamine, cytokines and eicosanoids such as prostaglandin D_2 ($PGD_{2)}$ (Dawicki and Marshall, 2007, Wedemeyer et al., 2000). It has been reported that UVR induces the migration of mast cells from skin to draining lymph nodes (Byrne et al., 2008).

1.1.3. Subcutaneous layer

The subcutaneous layer is situated below the dermal layer (Figure1.1). It is composed of connective and adipose tissue which is connected with the dermis's connective tissue. Through this layer the skin is stabilised and connected to the underlying body structure. At the same time, this subcutaneous layer separates the skin from the deep fascia, which is situated around the muscles (Premkumar, 2004).The adipose tissue serves as an energy storage reservoir and insulator.

In addition to all these cells, skin also has neural, vascular and lymphatic systems, sensory nerve receptors, free terminals and hair follicle endings. Extensive vasculature housed in the dermis helps to regulate body temperature, to deliver oxygen and nutrients, and to remove toxins and waste products. Skin also has many glands such as the sebaceous gland, which is responsible for secreting sebum which serves to maintain the pH of the skin surface at around

5. Another major gland in the skin is the sweat gland, which is found in almost every part of the skin and produces sweat. Sweat production is a control mechanism useful for maintaining the homeostasis of the body.

1.2. Ultraviolet radiation and human skin

The sun is the main source of UVR reaching the surface of the earth. More than 98% of UVR in the sunlight is blocked by the earth's ozone layer. The wavelength range of UVR is longer than X-ray-s and shorter than visible light (400 – 700nm), ranging, typically from 100 to 400 nm (Figure 1.3). The UVR band can be subdivided into three ranges: UVA 315 – 400 nm, UVB 280 – 315 nm and UVC 100 – 280 nm (Ghetti et al., 2006, Ohnaka, 1993). The ozone layer is able to absorb most UVR with a wavelength of less than 295 nm. Therefore, UVC does not reach the earth's surface and only UVB and UVA are important in respect to human skin. Depending on the type and amount of UVR, humans can experience either a beneficial or a harmful effect (Ohnaka, 1993).

UVB is considered the most destructive form of UVR because it is plentiful in the environment. UVB radiation is mainly absorbed by the epidermis and upper dermis. However, UVA, with its longer wavelength can penetrate deeper through the epidermis to reach the dermis (Costin and Hearing, 2007). UVB has enough energy to cause direct damage to cellular DNA. Harmful effects can include erythema, cataracts, and the development of skin cancer. UVA can also cause DNA damage primarily through the generation of reactive oxygen species (ROS) (Kvam and Tyrrell, 1997). Moreover, both UVA and UVB stimulate the production of melanin and induce apoptosis of melanocytes and keratinocytes an effect that has been recognised as a protective mechanism in the skin (Bowen et al., 2003). In general, UVB and UVA contribute to freckling

and skin wrinkling and can induce skin carcinogenesis in a number of ways: directly by generation of DNA photoproducts or indirectly by releasing mediators that cause oxidative damage to DNA (Ravanat et al., 2001, Lee et al., 2013a).

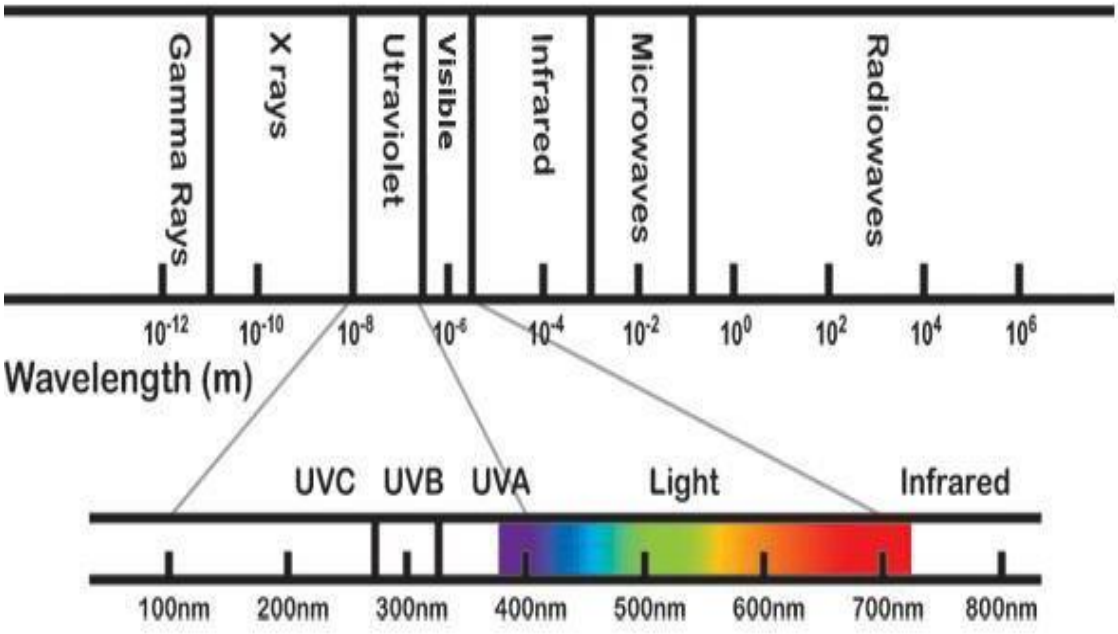


Figure.1.3. Wavelength ranges of the electromagnetic radiation spectrum (Stroebel and Richard. 2009)

1.2.1. Effect of ultraviolet radiation on DNA

DNA absorbs UVR with a wavelength of about 260nm and the absorption is decreased at increased wavelength up to about 300nm. This wavelength range is within the UVB range, and UVB is responsible for direct DNA damage in the cells (Taylor, 1994). The cyclobutane pyrimidine dimers (CPD) and thymine dimers are the major DNA photoproducts generated after absorbance of UVR. CPD and the (6-4) pyrimidine pyrimidon photoproduct ((6-4) (PPs) have a role in the development of skin cancer. Specific mutations from these

products lead to UVR fingerprint which includes CC⟶TT and C⟶T (Ravanat et al., 2001). It has been suggested that formation of dimers especially CPD, by UVR led to keratinocytes releasing immunosuppressive mediators such as IL-10 and TNF-α (Walker and Young, 2007). The repair mechanism of CPD leads to stimulation of skin pigmentation which protects the DNA from further damage (Bestak and Halliday, 1996).

1.2.2. Effects of ultraviolet radiation on photoimmunosuppression

UVR has the ability to inhibit the immune system of the skin not only locally but also systemically. Local immunosuppression relates to the circumstance where the antigen is applied to the irradiated skin site, while during the process of systemic immunosuppression the antigen is away from a skin site that has been irradiated (Chung et al., 1986). High doses of UVB are needed to reduce immunity at distant non-exposed sites, while much lower doses are required to induce local photoimmunosuppression. Furthermore, it is recognised that local photoimmunosuppression by UVB is caused even at suberythemal doses (Young, 2003, Vink et al., 1996). Various mechanisms may be involved in systemic and local immunosuppression. On the other hand UVR has a restricted ability to penetrate the skin and generates systemic immunosuppression through stimulation of soluble mediators and cytokines, such as IL-10, IL-4 and IL -13, (Schwarz et al., 1996, Loser et al., 2007) and tumor necrosis factor alpha (TNF-α) (Werth et al., 2003) and lipid mediators such as platelet activating factor (PAF) and prostaglandin E_2 (PGE_2) .

With regard to both systemic and local immunosuppression, it is recognised that the immune system can be compromised by UVR in an antigen-specific way through the creation of immune tolerance; antigen specific Tregs

may be identified in the draining LNs of the skin, as well as the spleen, after UVR (Schwarz, 2008, Schwarz, 2005). The immunosuppressive impacts of UV-exposure in the specific case of humans can be seen through various examples, including reduced contact hypersensitivity (CHS) responses, as well as the re-activation of herpes simplex as a result of direct sun exposure (Damian et al., 2011).

1.2.3. Effect of ultraviolet radiation on urocanic acid

Studies on the UVR effects and absorption by the components of the skin indicated that in addition to DNA, urocanic acid (UCA) is an important photoproduction in the skin. UCA is synthesised by keratinocytes during the process of keratinisation (Noonan and Defabo, 1992). After interaction with UVR, *trans*-UCA, which is the major skin UCA isomer, is converted to *cis*-UCA isomer (De Fabo and Noonan, 1983). Several studies suggest that *cis*-UCA is one of the immunosuppression mediators induced by UVB (Noonan and Defabo, 1992, McLoone et al., 2005). Morover, it has been found that *cis*-UCA has a significant effect on both local and systemic CHS immunosuppression after UVR (Vanstrien and Korstanje, 1995). The mechanism of *cis*-UCA action is still unclear but it has been reported to act on cells through histamine-like receptors or platelet activating factor receptor (Beissert et al., 1997, Walterscheid et al., 2006). Finally, upregulation of the immunomodulatory mediators such as PGE_2, TNF-α and IL-6 was found after human keratinocytes were treated with *cis*-UCA (Kaneko et al., 2009).

1.2.4. Activation of apoptosis by ultraviolet radiation

Apoptosis is a protective mechanism that minimises and prevents the malignant transformation of cells with irreparable DNA damage. Apoptosis can be induced by protein p53 (p53), which activates transcription by up-regulating the expression of pro-apoptotic genes such as Bax and Fas. Also, p53 down-regulates the expression of anti-apoptotic genes such as Bcl-2. Over all, apoptosis in response to UVR induced damage is regulated at least in part by p53, p2, Bax and Bcl-2 pathways.

The effect of UV on the induction of apoptosis may depend on the dose and type of cell. Figure 1.4 shown an outline of the main ways of UVR induced apoptosis: (i) following DNA damage, p53 is activated and leaks to mitochondria cytochrome c. (ii) UVR can activate several cell membrane death receptors which then activate the caspase pathways. (iii) Activation of membrane death receptors by UVR leads to activation of caspase 8, which cleaves cytosolic Bid to its truncated active form (tBid). tBid can then be transferred to the membrane of mitochondria. This would induce the translocation and the oligomerisation of Bax or Bak. These proteins activate the release of cytochrome C from the mitochondria (Bang et al., 2003, Ashkenazi and Dixit, 1998). (iv) Finally, UVR can induce apoptosis by increase ROS generation, which can than change the structure and function of several molecules (Lee et al., 2013). All these apoptotic pathways are balanced by activation of the E2F1 transcription factor, which is up regulated in response to DNA damage and promotes DNA repair (Berton et al., 2005).

In the epidermis, apoptosis plays an important role in controlling the development of erythema which occurs after sun exposure (Kraemer, 1997).

The peak sunburn appears 24h post UVR exposure and within several days it gradually declines (Okamoto et al., 1999). The dead cells may be replaced by hyperproliferative cells, leading to epidermal hyperplasia. This implies that UV-induced apoptosis and hyperplasia are closely linked, tightly regulated and that deregulation of these pathways may lead to skin cancer development (Melnikova and Ananthaswamy, 2005).

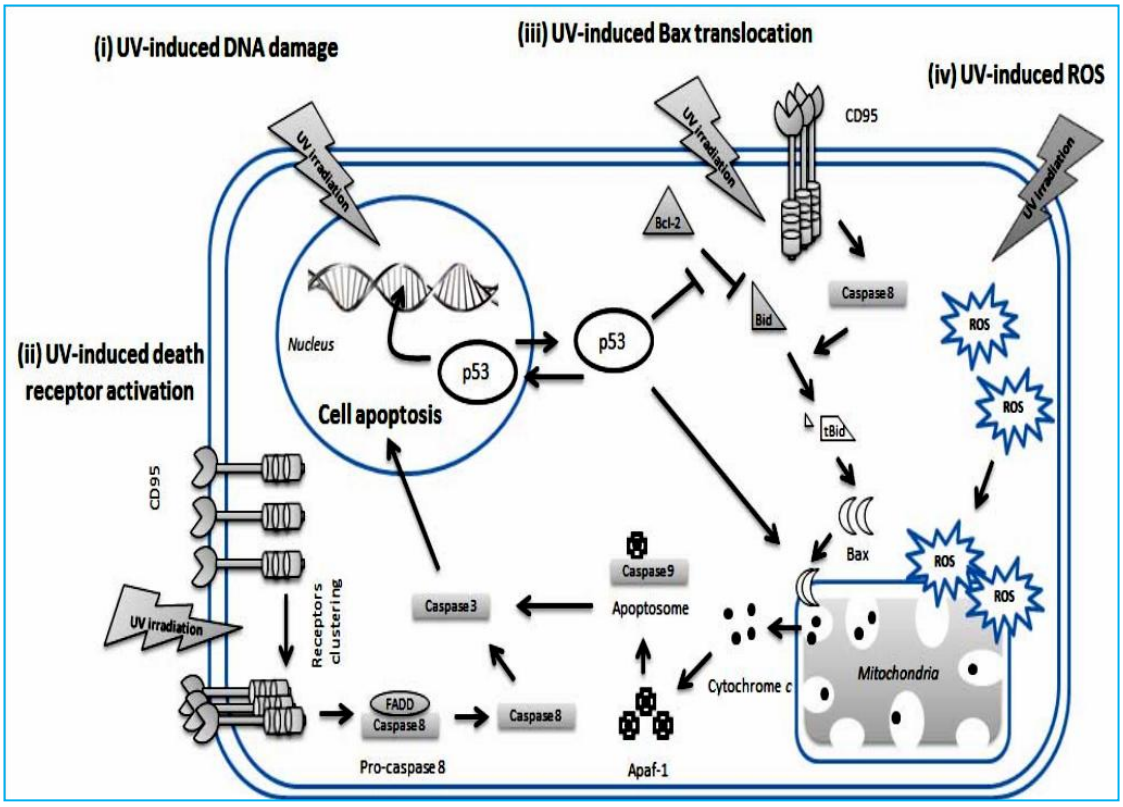


Figure.1.4. Mechanism of UVR induced apoptosis (taken from Lee et al., 2013a). (İ) UVR induced DNA damage, p53 is activated and leaks to mitochondria cytochrome c. (İİ) UVR can activate a membrane death receptors (extrinsic pathway). (İİİ) Activation of membrane death receptors by UVR leads to activation intrinsic pathway. (iv) UVR induces apoptosis by increase ROS generation.

Apaf-1= Apoptotic protease activating factor 1

CD95= Cluster of differentiation 95

FADD = Fas associated death domain

1.2.5. Skin pigmentation and ultraviolet radiation.

Pigmentation is one mechanism of skin protection against UVR. Through the barrier of keratinocytes that contain melanin, melanin functions as a photoprotector by absorbing UVR (Thody et al., 1993). There are many variations in human skin pigmentation including racial, regional, and individual, and these are affected by environmental factors such as different degrees of exposure to UVR. Skin colour depends on constitutive pigmentation and facultative pigmentation. Based on this skin has been classified into six skin prototypes, as shown in Table1.1 (Fitzpatrick, 1988). The classification is based on the ability of individuals to undergo melanogenesis (tanning) in response to sun or UVR exposure. Skin type I refer to individuals who always burn easily and do not tan in response to UVR. Meanwhile skin type VI represents people who are deeply pigmented, have very dark skin and almost never burn when exposed to UVR.

Table.1.1. Classification of human skin phototypes (Fitzpatrick, 1988).

Skin type	**Skin colour**	**Sunburn**	**Tanning**	**Skin cancer**
I	White	High	Very poor	High
II	White	High	Poor	High
III	Light Brown	Moderate	Good	Moderate
IV	Moderate Brown	Low	Very good	Low
V	Dark Brown	Very low	Very good	Very low
VI	Black	Never	Deep pigmentation	Very low

1.2.6. Skin response to acute UVR exposure

The acute effects of UVR from natural (sunlight) and artificial (lamps) sources are a major worry for human health. The major acute effects of UVR on normal human skin are: sunburn inflammation (erythema), tanning, and local or systemic immunosuppression (Matsumura and Ananthaswamy, 2004).

Erythema is an acute injury that occurs after extreme exposure to UVR. When the superficial blood vessels of the dermis dilate, the rate of blood flow increases in the skin, and this leads to redness of the skin (Diffey, 1991). This is apparent from about four hours after exposure and is maximal at about 24 hours (Young et al., 1996, Rhodes et al., 2009). Excessive exposure to UVR may lead to epidermal and dermal oedema, pain, and even blistering (Taylor and Sober, 1996).

Immediate tanning occurs within 5-10 min of UVR exposure and lasts for 12 hours before fading. Delayed tanning persists for weeks to months and occurs as a consequence of melanocytes stimulation and production of new melanin (Diffey, 1991).

Hyperplasia of the epidermis also occurs as a result of repeated exposure to the sun, which stimulates epidermal cell hyperproliferation and produces epidermal thickening. Thickening of the stratum corneum layer increase three-fold within one to three weeks, and about three fold- to five-fold within seven weeks. Skin thickness returns to normal about one to two months after exposure to irradiation has ceased. The increased thickening of the skin allows better protection against UVR (Diffey, 1991, Soter, 1990).

1.2.7. Skin response to chronic UVR exposure

It is clear that any excessive exposure to UVR can lead to damage of the human skin structure (Leyden, 1990). In the long-term, this damage can lead to photoageing and carcinogenesis, through chronic inflammation and DNA damage. Photoaging or dermatoheliosis refers to the structural changes in the skin resulting from frequent sun (UVR) exposure rather than the passing of time alone (Taylor and Sober, 1996). There are many clinical signs of photoaging such as, dryness, deep wrinkles, accentuated skin furrows, sagging, loss of elasticity, mottled pigmentation and telangiectasia (Leyden, 1990).

Photoaging appears in the epidermis as epidermal hypertrophy with keratinocyte disarray and hypertrophy of sebaceous glands and the dermal matrix (Young, 1990). Photoaging also leads to an increase in dermal thickening due to the presence of massive quantities of thickened, tangled and degraded elastic fibres. Overall, UVR is a complete carcinogen, able to initiate and promote cancer, DNA damage and immunosuppression (Ziegler et al., 1994).

1.3. Fatty Acids

Fatty acids are carboxylic acids that contain between 2-30 carbons. The hydrocarbon chain terminates with a carboxyl group at one end and methyl group at the other. The carboxyl group can form ester links with alcohols, such as cholesterol forming cholesterol esters, and glycerol forming various acylglycerols. Fatty acids with carbon chain lengths of between 12 and 22 are the most common in animal cells. Fatty acids can be saturated or unsaturated depending on the presence of double bonds in the carbon chain. Unsaturated fatty acids can be divided into two groups: monounsaturated fatty acids (MUFAs)

which contain only one double bond and polyunsaturated fatty acids (PUFAs) which contain two or more double bonds. PUFAs are also described by the position of the last double bond relative to the terminal carbon of the acyl chain. Following a popular shorthand notation, omega -3 polyunsaturated fatty acids that have the last double bond on the 3rd carbon from the end of the acyl are described as n-3 PUFA. Omega 6 polyunsaturated fatty acids with the last double bond on the 6th carbon from the end of the acyl chain are described as n-6 PUFA.

1.3.1. Fatty acid biosynthesis

Fatty acids are supplied through diet which is considered the main and most important source for the provision of essential fatty acids. However, many fatty acids are produced by endogenous synthesis that occurs mainly in the liver. Fatty acid biosynthesis takes place in the cytosol starting with acetyl-CoA which is formed from pyruvate or by β-oxidation of the fatty acids in the mitochondria. Acetyl-CoA reacts with oxaloacetate to give citrate, which has to be transported from the mitochondria to the cytosol where it is transformed back to acetyl-CoA. Following the reaction catalysed by acetyl-CoA carboxylase, malonyl-CoA is formed and the acyl chain starts growing by the addition of 2-C up to a length of 16C (palmitic). This occurs with the help of the multi functioning enzyme fatty acid synthase (Mayne, 1994).

There are some tissues which contain enzymes that produce short chain saturated acids: for example, the mammary gland of some species can produce fatty acids containing 8 to 10 carbons. The biosynthesis of fatty acids longer than palmitic acid occurs through additional reactions leading to elongation and desaturation of the acyl chain.

1.3.2. Biosynthesis of unsaturated fatty acids

Most monounsaturated fatty acids are synthesised by the addition of a single double bond to a saturated fatty acid this reaction occurs typically between carbons number 9 and 10 by the action of the enzyme delta (∆) 9 desaturase. For example, stearic acid (C18:0) is transformed into the monounsaturated fatty acid oleic acid (OA,18:1n-9) (Figure 1.5). All eukaryotes and some bacteria can form PUFA. Plants can synthesise PUFA by adding a new double bond between the existing double bond and terminal methyl group, whereas animal desaturases normally introduce a new double bond between an existing double bond and the carboxyl group. Insertion of a new double bond between carbons 12 and13 (counted from the carboxyl carbon) of OA yields linoleic acid (LA,18:2 n-6) and the enzyme that catalyses this reaction is called ∆ 12 desaturase (Figure 1.5). LA yields α-linolenic acid (ALA,18:3 n-3) by the addition of a double bond between carbons 15 and 16 by ∆ 15 desaturase (Minich, 1999, Newsholme and Leech, 2009). The human body cannot produce fatty acids which contain at least two double bond positioned from C9 and above, because the ∆ 12 and ∆ 15 desaturases are absent. As a result LA and ALA cannot be synthesised *de novo* and must be obtained from the diet (Edwards and O'Flaherty, 2008). LA and ALA are known as essential fatty acids. LA is the parent fatty acid of the n-6 PUFA family and ALA is the parent fatty acid of the n-3 PUFA. The pathway of desaturation and elongation occurs in the liver. LA is converted to γ -Linolenic acid (GLA, 18:3 n-6) by ∆ 6 desaturase and is then elongated to dihomo γ-linolenic (DGLA, 20:3 n-6). Arachidonic acid (AA, C20:4 n-6) is generated from DGLA by a ∆ 5 desaturase. A summary of these biosynthetic pathways is shown in Figure 1.5.

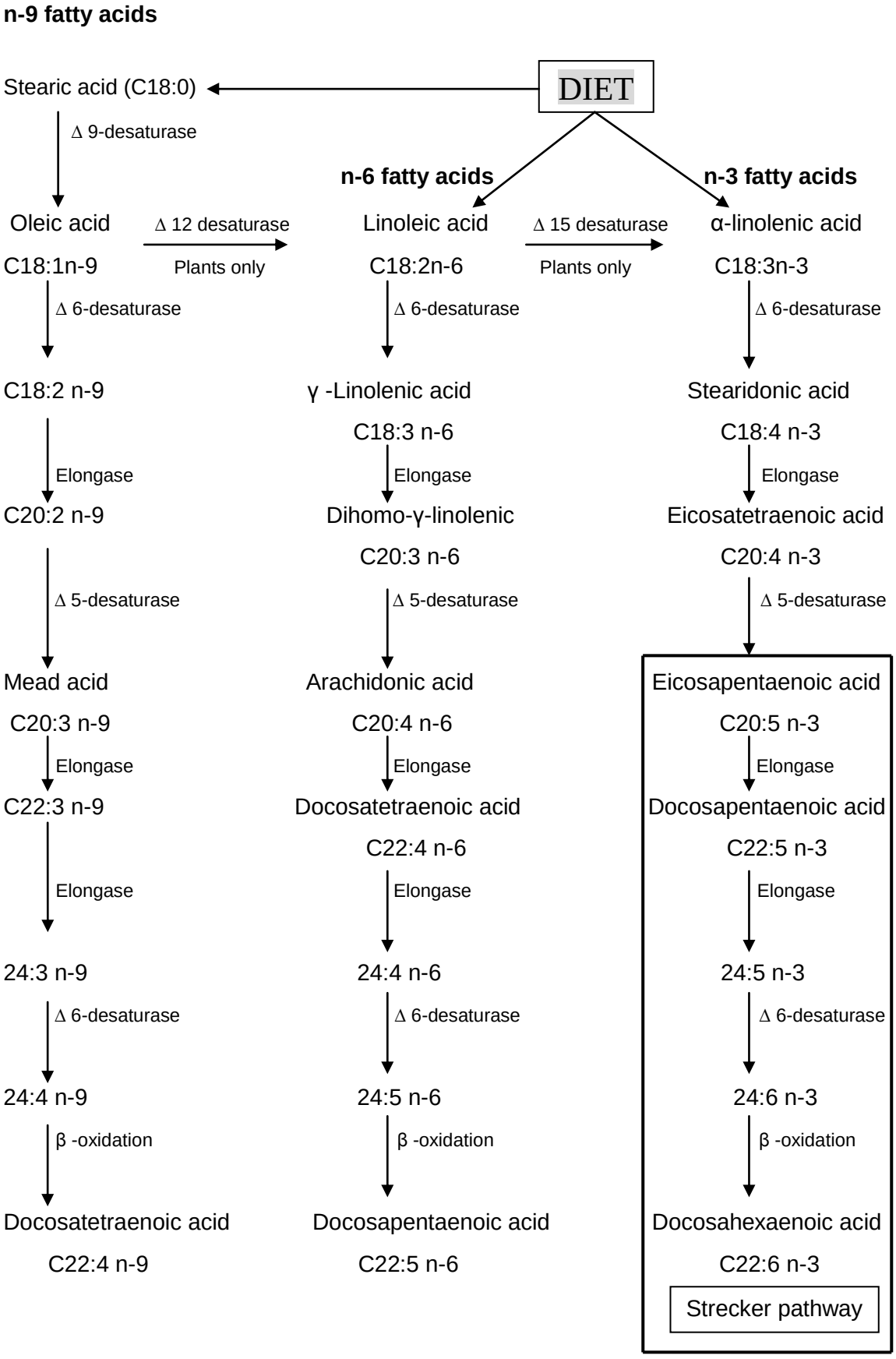


Figure.1.5. Outline of the major pathways involved in the biosynthesis of polyunsaturated fatty acids

1.3.3. Omega-3 polyunsaturated fatty acids

Dietary n-3 PUFAs have properties important for health and disease, such as cardiovascular neuronal development, and regulation of plasma lipid levels and immune function (Aarsetoey et al., 2012). Intake of n-3 PUFAs leads to their distribution to virtually every cell in the body, with effects on membrane composition and function, eicosanoid synthesis, and signalling, as well as the regulation of gene expression(Salem et al., 2001). The n-3 PUFAs that are important in human nutrition are: ALA, EPA and DHA (Lands, 1992). Several animal studies reported that the relative levels of n-6 and n-3 PUFA can be regulated by altering the balance of LA and ALA in the diet (Blank et al., 2002). The endogenous synthesis of n-3 PUFA from ALA is independently regulated by transcription factors or changes in the expression of the biosynthetic enzymes and also depends on substrate competition for existing enzymes rather than an increase in their mRNA expression (Tu et al., 2010). It has also been reported that a diet rich in LA can lead to inhibition of DHA synthesis (Gibson et al., 2013). Oily fish such as mackerel, salmon, sardines, anchovies and herring are the main sources of the long chain n-3 PUFA EPA and DHA. Lower amounts of n-3 PUFA are found in other fish such as tuna. Vegetable oils such as soybean and canola are a rich source of ALA. (Gerster, 1998). In addition to diet, endogenous synthesis is another source of n-3PUFA. EPA is elongated to DPA, further elongated to C24:6 n-3 and through the β oxidation is converted to DHA. The levels of DPA in circulation correlate with EPA level not with the fish consumption, in contrast high DHA levels correlate with dietary consumption, not with endogenous synthesis (Pawlosky et al., 2001). This

means that DPA comes from exogenous EPA and DHA, both of which depend on nutrition.

1.4. Eicosanoids and related lipid mediators

Eicosanoids were first identified by Kurzrok and Leib of Columbia University in 1930, as a chemical that had an effect on cervical muscle cramps. In the mid-1930s, Euler discovered that human semen contained active compounds which, when injected, can contract or relax smooth muscle and affect blood pressure (Kurzrok. and Lieb., 1930). The biosynthetic pathways for eicosanoid production were discovered by Bergstrom, Samuelsson, and their associates in the early 1960s (Bergstrom and Sjovall., 1962). Eicosanoids are autacoids that are not stored but produced when needed (Velzing-Aarts et al., 1999, Yilmaz, 2001). The eicosanoids have physiological effects on many biological systems such as the cardiovascular system, brain, eye, lung, kidney and skin (Bishop-Bailey et al., 2002, Ishihara et al., 2004).

There are two main families of eicosanoids: prostanoids which include prostaglandins, prostacyclins, and thromboxanes, and the leukotrienes (Velzing-Aarts et al., 1999). Oxidation of the C20:0 PUFA AA, EPA and DGLA, generates eicosanoids. When the parent fatty acid has 22 carbons (22 = docosa), the family of lipid mediators produced is termed docosanoids. When the precursor fatty acid has 18 carbons (18 = octadeca) the family is called octadeconoids. Three groups of enzymes contribute to the formation of eicosanoids and related lipid mediators: cyclooxygenases (COX-1 and COX-2), lipoxygenases (LOX) and cytochrome P450 oxidases (Funk, 2001, Ciccoli et al., 2005).

Biosynthesis of eicosanoids depends on the availability of free PUFA which depends on the capacity of the enzymes that remove them from membrane phospholipids. It is also influenced by dietary intake. Increased intake of foods rich in n-3 PUFA tends to decrease membrane AA content in favour of increased n-3 PUFA. Eicosanoid synthesis is precisely controlled. An effective way of regulation is through the expression of eicosanoids biosynthetic enzymes, which can be either down-regulated or up-regulated (Brock and Peters-Golden, 2007).

1.4.1. The role of phospholipases in lipid mediator production

Phospholipases (PL) have an important role in eicosanoid and lipid mediator production. The first step in the biosynthesis of eicosanoids is the hydrolysis of membrane glycerophospholipids to release AA and other PUFA (Figure 1.6). There are many types of phospholipases but phospholipase A_2 (PLA_2) is the most relevant enzyme (Dennis, 1987). The production of free AA is highly regulated and is balanced between two competing reactions, reacylation and transfer into various phospholipid pools by acyltransferases and transacylases, and phospholipid hydrolysis by PLA_2 (Chilton et al., 1996).

More than 15 different mammalian PLA_2 enzymes have been identified and they differ in their intracellular localisation and biochemical characteristics. The three major types are: secretary phospholipase A_2 ($sPLA_2$), cytosolic Ca^{2+} dependent phospholipase A_2 ($cPLA_2$) and Ca^{2+} independent cytosolic phospholipase A_2 ($iPLA_2$)(Dennis, 1994).

Among the PLA_2 types, $sPLA_2$ is a secretable isoform, with a low molecular mass of 13-18 kDa and it requires a millimolar concentration of Ca^{2+} for catalytic activity (Fuentes et al., 2002, Chen et al., 1994). Several $sPLA_2$ subtypes have

been described in various cells: $sPLA_2$ isoforms have been detected in keratinocytes but at low level mRNA including; $sPLA_2$-s-IIA, -IID, -V, and –X. However, only $sPLA_2$-X has been identified as being secreted by keratinocytes (Schadow et al., 2001). It has also been reported that $sPLA_2$-X stimulates both tyrosinase activity and dendricity in melanocytes and this is mediated by lysophosphatidylcholine (Scott et al., 2006). Ca^{2+}independent cytosolic phospholipase A_2 ($iPLA_2$), does not require Ca^{2+} for catalytic activity. $iPLA_2$ is similar in size (83-85 kDa) and shares the same intracellular location with $cPLA_2$ (Six and Dennis, 2000). Although Ca^{2+} is not required for the enzymatic action of $iPLA_2$, it is reportedly up-regulated by calcium or calcium-dependent factors in some cell models (Murakami and Kudo, 2004).

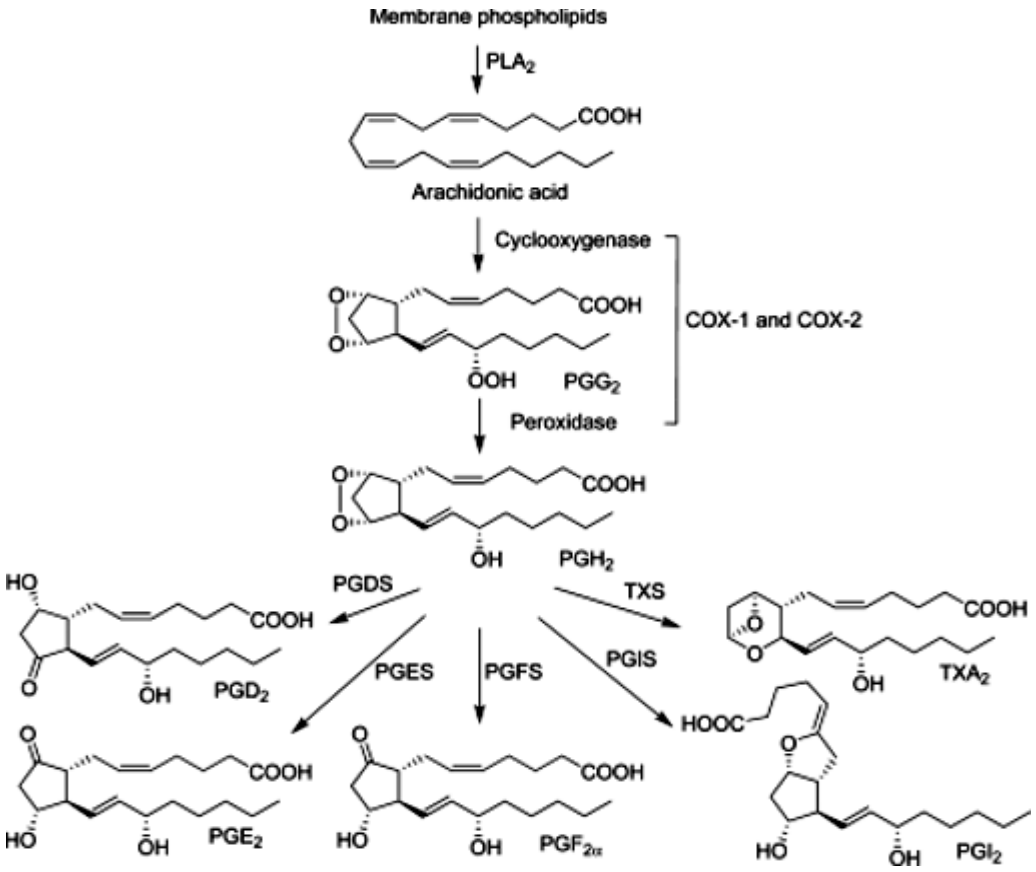


Figure.1.6. Outline of the biosynthetic pathway of prostanoids (taken from Waltenberger et al., 2011)

Cytosolic Ca^{2+} dependent phospholipase A_2 ($cPLA_2$) has a molecular mass of 85 kDa and is present in many cells and tissues. $cPLA_2$ was first discovered in platelets and macrophages. Ca^{2+} is required for $cPLA_2$ activation and is necessary for binding to membranes (Kramer and Sharp, 1997, Clark et al., 1995). $cPLA_2$ plays an important role in releasing AA specifically from the sn-2 position of membrane phospholipids for eicosanoids production (Balsinde et al., 1995) and also has a role in the lysophospholipid regulation. $cPLA_2$ has been considered a target for the development of anti-inflammatory drugs. $cPLA_2$ contributes to the long-term generation of AA preceding eicosanoid production in differentiated human keratinocytes (Sjursen et al., 2000).

1.4.2. Prostanoids

The term prostanoids is used to describe the following eicosanoids: prostaglandins (PG), prostacyclin (PGI), and thromboxanes (TX). All prostanoids are derived from the unstable PGH_2 through the action of many different prostanoid syntheses (Figure1.6). Prostanoids are involved in the inflammatory response. Prostaglandins are oxygenated C20-PUFA that contain a five-member ring; in prostacyclin there is a bridge of oxygen between the C6 and C9 and thromboxane has a bicyclic oxane-oxetane ring structure. TXA_2 is unstable and converts to the stable TXB_2. The number of double bonds in prostanoids depends on the precursor of fatty acid. Series-1 prostanoids have only one double bond and derive from DHGLA. Series-2 prostanoids have two double bonds and derive from AA. The series-3 prostanoids have three double bonds and derive from EPA. In general, AA-derived eicosanoids are considered to have pro-inflammatory effects, whereas EPA and DHGLA derived eicosanoids have anti-inflammatory effects (Cowing and Saker, 2001). Figure

1.7 shows the structures of three types of prostaglandins E (PGE) and prostaglandins F (PGF) and their precursor PUFA.

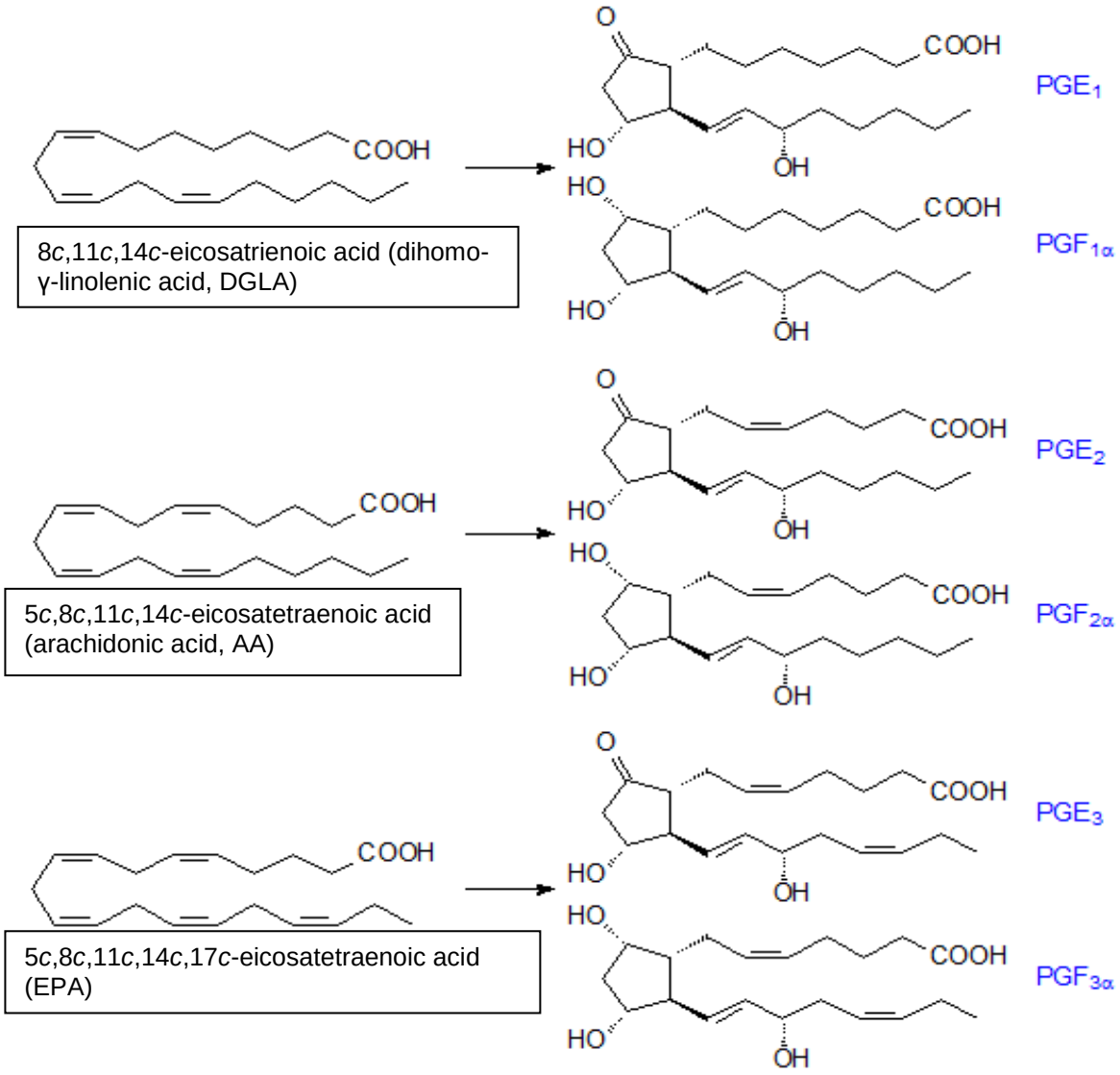


Figure.1.7. Structures of series 1,2 and 3 of PGE and PGF and their precursor polyunsaturated fatty acid (taken from lipidlibrary.aocs.org)

1.4.2.1. The role of cyclooxygenase in prostanoid production

Cyclooxygenase (COX) was first purified in 1976 and cloned in 1988. It is the key enzyme in the biosynthesis of prostaglandins (Vane et al., 1998). There are two isoforms of COX; cyclooxygenase-1 (COX-1) and cyclooxygenase-2

(COX-2). The enzymes are also known as endoperoxide prostaglandin H synthases-1 (PGHS-1) and -2 (PGHS-2). The structure of the active site in both COX-1 and COX-2 is very similar. However, it is larger in COX-2 than in COX-1 (Smith et al., 2000). COX-1 is constitutively expressed in cells that produce prostaglandins involved in homeostasis. COX-2 is an inducible enzyme and can be up regulated in response to various stimuli. The presence of hydroperoxide at catalytic level is required for cyclooxygenase reaction (Kulmacz et al., 1994). However, COX-2 can be activated at 10 times lower hydroperoxide concentration than COX-1 (Tsai et al., 1995). In addition to AA, both COX-1 and COX-2 can metabolise EPA and DGLA to form prostanoids. The COX reaction adds two molecules of oxygen to AA to form prostaglandin G_2 (PGG_2). Then, by a peroxidase reaction PGG_2 is converted to prostaglandin H_2 (PGH_2) which is very unstable. Once PGH_2 is produced it is converted to a number of important biological mediators such as PGE_2, PGD_2 and PGI through the action of prostanoid synthases (Tsai et al., 1995, Kurumbail et al., 2001, Malkowski et al., 2001) (Figure 1.6).

Cyclooxygenase can be inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen. Among all NSAIDs only aspirin can modify both COX-1 and COX-2 and inhibit it by acetylation of a serine residue positioned in the catalytic site of the enzyme (to Ser^{529} of COX-1 and Ser^{516} of COX-2). Inhibition or inactivation of COX leads to the inhibition of PGs and TX production (Loll et al., 1995).

1.4.2.2. The role of prostanoid synthases

Prostaglandin D synthases (PGDS) catalyse the isomerisation of the endoperoxide PGH_2 to PGD_2. Two types of PGDS have been identified: the

haematopoietic type PGDS (H-PGDS) and the lipocaline type PGDS (L-PGDS) (Urade and Hayaishi, 1999).

Prostaglandin E synthase (PGES) forms PGE. Three types of PGES have been characterised: the cytosolic PGES (cPGES) (Tanioka et al., 2000) and two isoforms of the membrane-bound PGES (mPGES) (Jakobsson et al., 1999): known as mPGES-1 and mPGES-2 (Tanikawa et al., 2002). In most tissues the generation of PGE_2 involved in cellular homeostasis occurs via the action of COX-1 and cPGES. However when PGE_2 is generated in response to a pro-inflammatory stimulus, this occurs via the coordinated up-regulation and functional coupling of COX-2 and mPGES (Stichtenoth et al., 2001, Mancini et al., 2001). This can occur by common signalling, such as nuclear factor kappa B (NF-kB). Furthermore, the expression of mPGES-2 is induced under pathological conditions (e.g. cancer) and interacts with COX-2 (Park et al., 2006, Samuelsson et al., 2007).

There are two isoforms of the prostaglandin F synthase (PGFS): PGFS-I (lung-type) and PGFS-II (liver-type) (Suzuki et al., 1999). However the biosynthesis of PGF_2 can occur via three pathways: directly from PGH_2, or from PGE_2 or PGD_2. PGH 9-,11-endoperoxide reductase (PGH 9,11ER) catalyses the formation of $PGF_{2\alpha}$ from PGH_2; PGE 9-ketoreductase (PGE9-KR) catalyses the formation of $PGF_{2\alpha}$ from PGE_2; and PGD 11-ketoreductase (PGD11-KR) catalyses the formation of 9α,11β-$PGF_{2\alpha}$ from PGD_2 (Suzuki-Yamamoto et al., 1999).

Prostacyclin (PGI_2) is formed from PGH_2 by the enzyme prostacyclin synthase (PGIS). PGI_2 is unstable and hydrolysed nonenzymatically to the inactive metabolite 6-keto-$PGF_{2\alpha}$ (Liou et al., 2000, Hara et al., 1994). PGIS is

widely expressed in human tissues particularly in the endothelial cells. Futhermore, in endothelial cells the expression and production of both PGIS-mRNA and PGIS-protein can by induced by TNFα and some inflammatory cytokines. Finally, thromboxane A synthase (TXAS) converts PGH_2 to TXA_2 which is also unstable and is nonenzymatically converted to TXB_2.

The cyclopentenone prostaglandin PGJ_2 is produced by spontaneous dehydration of PGD_2. Similarly, PGJ_2 isomerises to form Δ^{12}-PGJ_2 and then goes through a secondary dehydration to15-deoxy-Δ12,14-PGJ_2.

1.4.2.3. The role of prostanoid receptors

Prostanoids have many functions and mediate physiological and pathophysiological processes in various organs, tissues and cells. They act via specific G-protein-coupled receptors. These are described as follows: DP for PGD, EP1, EP2, EP3 and EP4 for PGE; FP for PGF; TP for TX; and IP for PGI (Breyer et al., 2001). In addition, there are two isoforms of FP (FP_A and FP_B), and TP (TP_α and TP_β), and eight of EP_3 which only different in their C-terminal and are generated through alternative splicing. Moreover, PGJ_2 and 15-deoxy-12,14-PGJ_2 (15d-PGJ2) activate the PPAR nuclear receptors. The effect of prostanoid receptor activation on cell function is mediated by different intracellular signalling pathways. EP2, EP4 and IP receptors are coupled to G-protein s (G_s) and can activate adenyl cyclase which increases production of intracellular cyclic adenosine monophosphate (cAMP). However, via G_q EP1 and FP activate phosphatidylinositol metabolism. Finally, TP acts through two types of G-protein, G_q and $G_{l3,}$ which activate phospholipase C. Although both TP isoforms are coupled to phospholipase C activation, TPα stimulates adenyl cyclase, whereas TPβ inhibits it (Hatae et al., 2002).

Prostaglandins can have opposing effects depending on their signal transduction pathways and specific receptors. For example, PGE_2 has vasoconstrictive effects when acting through EP1 or EP3 receptors but it has a vasodilator effect when acting via EP2 or EP4 receptors (Tang et al., 2000). Also, PGE_2 can be pro-inflammatory and anti–inflammatory depending on its interactions with one of the EP receptors (Gualde and Harizi, 2004). Moreover, a low level of PGE_2 decreases proliferation of human keratinocytes by stimulating EP3 receptor, whereas a high level of PGE_2 stimulates proliferation by activation of the EP2 receptor. It has been found that deletion of EP2 receptor can protect against UVR induced carcinogenesis, but it increases tumour aggressiveness (Brouxhon et al., 2007).

1.4.2.4. Prostanoid deactivation

Prostanoids are locally formed and function close to the site of synthesis. They are deactivated within the same or neighbouring cells before they are released into the circulation as inactive metabolites. These catabolic mechanisms are important for the regulation of biological activities. There is one catabolic pathway that is common for most prostanoids and occurs via two steps: a) The PG uptake across the membrane by a carrier prostaglandin transporter (PGT). b) When the PG enters the cell, cytoplasmic oxidation to the 15(s)-hydroxyl group by the 15-hydroxyprostaglandin dehydrogenase (15-PGDH) (Figure1.8). 15-keto- and 13,14-dihydro-15-keto PGs are formed as a result of hydrogenation and reduction reactions (Tai et al., 2002). It has been reported that about half of PGE_2 that is taken up by the epithelial monolayer is oxidized to 13, 14-dihydro, 15-keto-PGE_2 (Nomura et al., 2004).

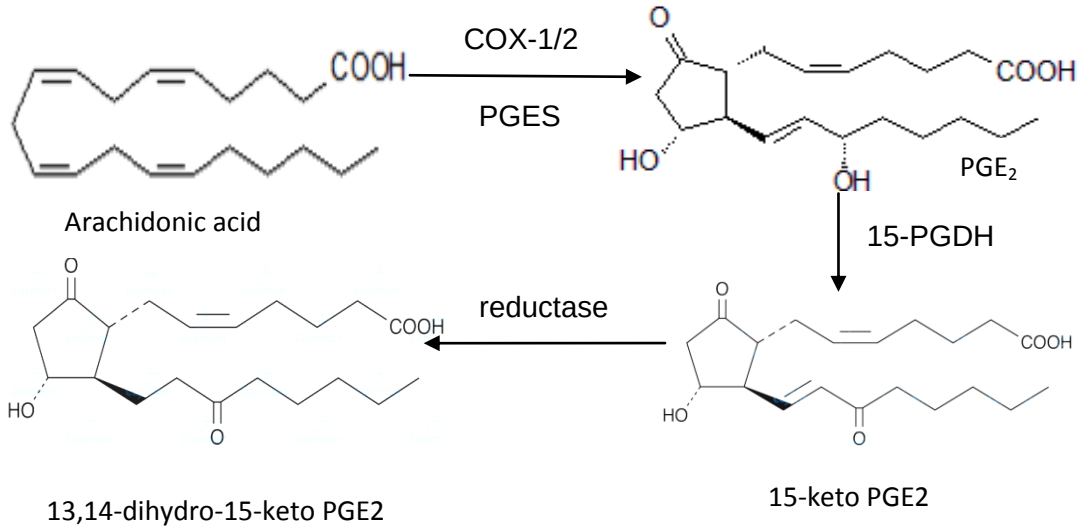


Figure.1.8. Schematic diagram of the deactivation pathway of PGE_2. COX-1/-2 =Cyclooxygenase -1/-2. PGES= Prostaglandin E synthase. 15-PGDH=15 – hydroxy-prostaglandin dehydrogenase.

1.4.3. Isoprostanes

The isoprostanes (IsoPs) are prostaglandin-like compounds formed through non-enzymatic pathways by free radical or reactive oxygen species (ROS) (Morrow et al., 1990). A number of peroxidation products are formed as a result of lipids being attacked by free radicals and AA is converted to PGH_2-like intermediates which are converted to prostaglandin-like compounds (isoprostanes) (Morrow et al., 1994). The most studied IsoPs are the F_2-IsoP that contain a ring type F similar to $PGF_{2\alpha}$. According to the position of the hydroxyl group in the chain the F_2-IsoP can be: 5-F_2-IsoP, 8-F_2-IsoP, 12-F_2-IsoP or 15-F_2-IsoP. Furthermore, in vitro prostaglandin F3-like compounds (F_3-IsoP) are generated from EPA (Gao et al., 2006) and prostaglandin F_4-like compounds (F_4-IsoP) from DHA (Musiek et al., 2004).

1.4.4. Lipoxygenase derived lipid mediators

Lipoxygenases (LOX) are a family of enzymes that oxygenate PUFA to specific hydroperoxide products. There are many types of LOX found in human

cells: 5-LOX, 12*S*-LOX, 12*R*-LOX, 15-LOX and the epidermal LOX type 3 (eLOX3) (Yu et al., 2003, Kinzig et al., 1999, Krieg et al., 2001). LOX act by the addition of two oxygens to polyunsaturated fatty acids to produce unstable hydroperoxides which are than reduced to hydroxy fatty acids such as leukotrienes (LTs), lipoxins (LXs) and other bioactive lipids (Kuhn and O'Donnell, 2006). Oxygenation of PUFA by LOX can generate an array of mono- and polyhydroxy fatty acids (Figure1.9): AA produces hydroxyeicosatetraenoic acids (HETEs), LTs, and LXs (Vance and Vance, 2002). Oxygenation of EPA by LOX generates hydroxy eicosapentaenoic acids (HEPEs) and E-series resolvins (RvEs). DHA produces hydroxy docosahexaenoic acids (HDHAs), D-series resolvins (RvDs), protectins (PDs) and maresins (MAR). LA forms hydroxyoctadecadienoic acids (HODEs) and DGLA forms hydroxyeicos-atrienoic acids (HETrE').

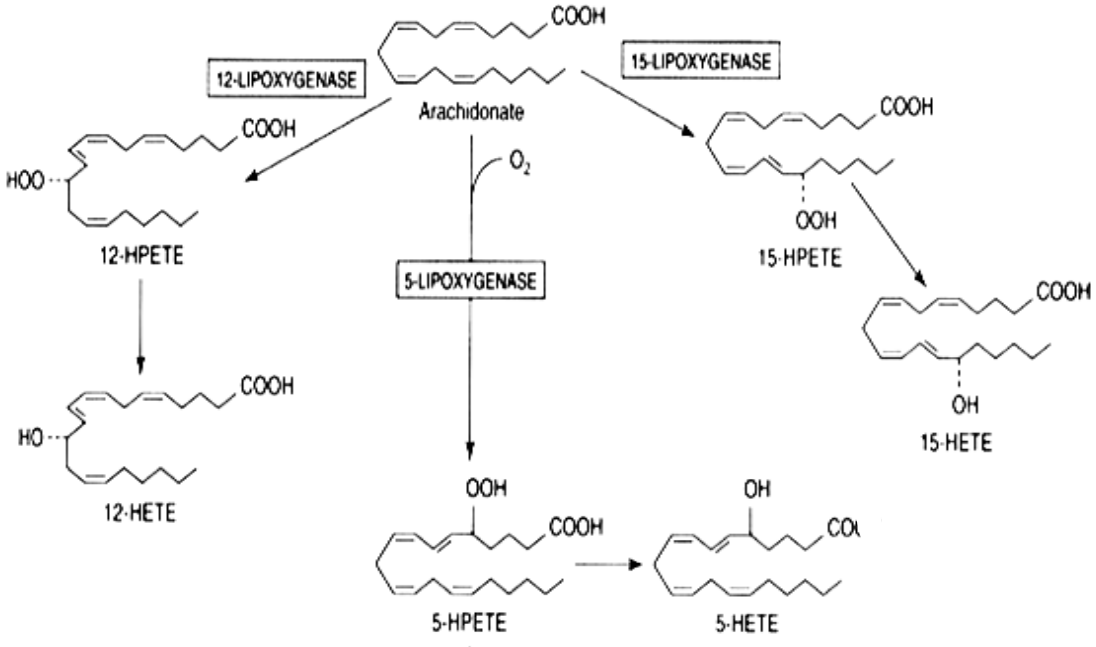


Figure.1.9. Schematic diagram of the biosynthesis of hydroxyl fatty acid (taken from www .lipidlibrary. aocs.org)

1.4.4.1. Leukotrienes

The name leukotriene derives from the original discovery of white blood cells (polymorphonuclear leucocytes). Leukotriene biosynthesis depends on the availability of free-AA. By the action of 5-LOX arachidonic acid is metabolised to 5(s)-hydroxyperoxyeicosatetraenic acid (5(S)-HPETE). The nuclear membrane protein, 5-lipoxygenase activating protein (FLAP) is required to translocate the cytosolic 5-LOX to the nuclear membrane (Ferguson, 2012, Woods et al., 1993). 5(S)-HPETE is rapidly reduced to 5(s)-hydroxyeicosatetraenic acid (5(S)-HETE). 5(s)-HETE is then metabolised to leukotriene A_4 (LTA_4). LTA_4 is unstable and readily forms LTB_4. LTB_4 is also conjugated with GSH to give the peptide leukotriene LTC_4 which has an important role in regulating acute inflammatory responses (Wada et al., 2006) (Figure 1.10). LTC_4 is transported out of the cells and metabolised to LTD_4, and then to LTE_4, which are then secreted in the urine (O'Donnell, 1999). LTA_4 can undergo non-enzymatic reactions to form diHETEs (Vance and Vance, 2002).

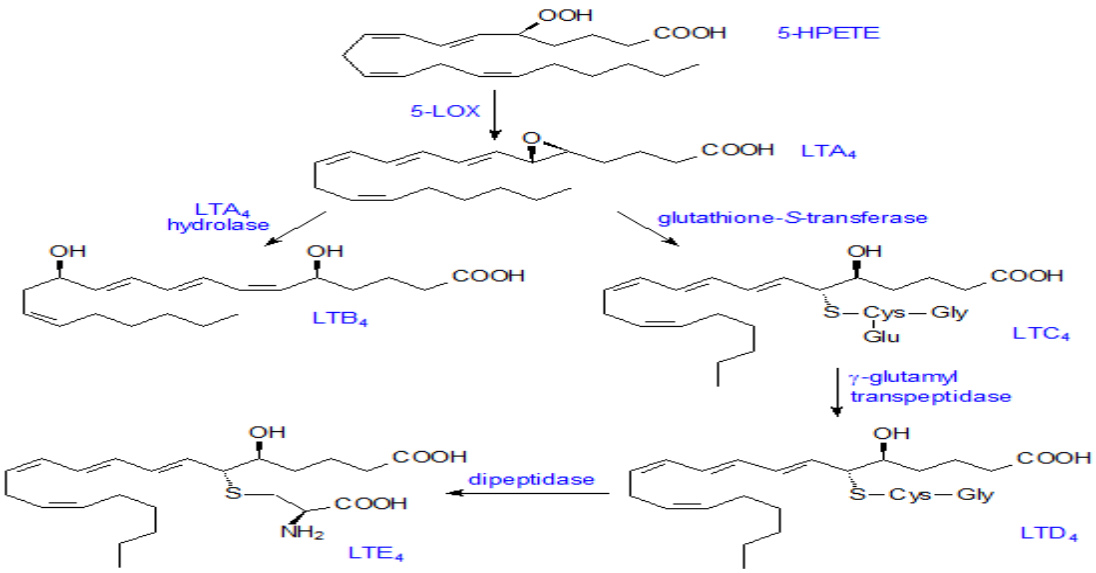


Figure.1.10 Schematic diagram of the biosynthesis leukotrienes (taken from www .lipidlibrary. aocs.org)

1.4.4.2. Lipoxins

Lipoxins are trihydroxy-eicosatetraenoic acids that derive from arachidonic acid (Serhan, 1994). They are structurally similar to leukotrienes and appear to have some complementary biological activities. The biosynthesis of lipoxins can occur by many biosynthetic pathways involving LOX activation as shown in (Figure 1.11). A) LTA_4 is generated by the action of 5-LOX, and then LTA_4 is reduced by the action of 12-LOX to generate LXA_4 and LXB_4. B) The 15-HPETE is generated by 15-LOX. Then, 5-LOX can convert 15-HPETE to a 15(6)-epoxy-tetraenoic intermediate that is further metabolised by LOX to form LXA_4 and LXB_4. The aspirin-triggered lipoxin 15-epiLXA_4 is formed throughout COX-2 and 5-LOX (Takano et al., 1997). LXA_4 is biologically active and, anti-inflammatory, and has recently been found to have neuroprotective effects in brain ischemia. LXA_4 also inhibits the up regulation of both LTB_4 and LTC_4 (Wu et al., 2012).

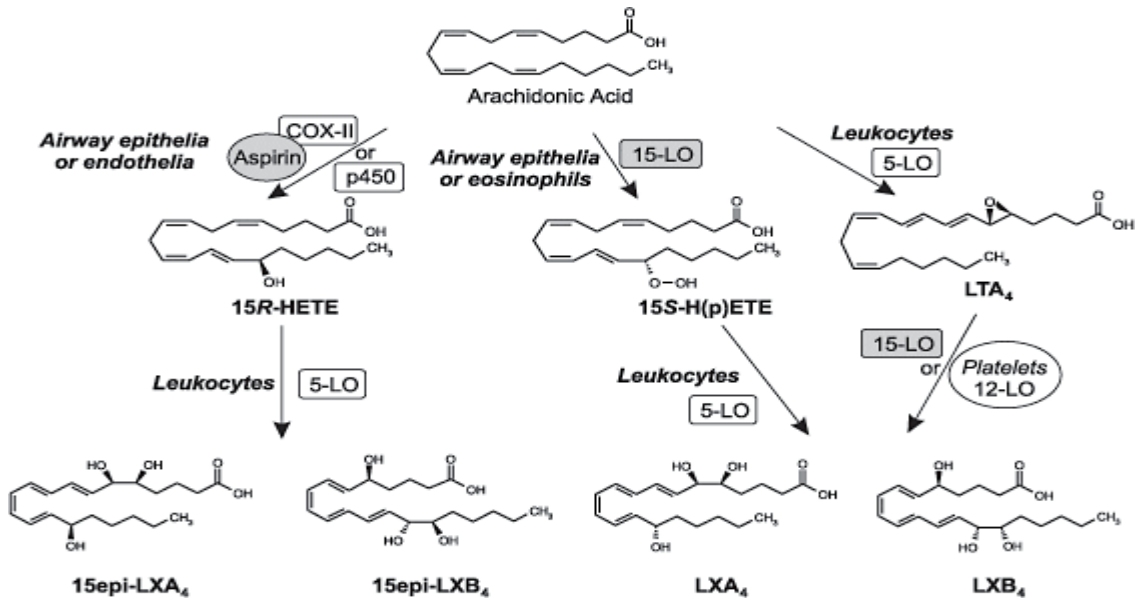


Figure 1.11 Diagram of the biosynthesis of lipoxins (Taken from Levy and Serhan, 2003)

1.4.4.3. Resolvins, Protectins and Maresins

Resolvins or ‘resolution-phase interaction products’ were discovered by Serhan et al in resolving inflammatory exudates (Serhan et al., 2002). Resolution of inflammation is the removal of leukocytes and debris from inflamed area, enabling the tissue the return to homeostasis. Rather than being a passive process, resolution is now considered an active biochemical and metabolic process. Compounds derived from EPA are resolvins of the E series (RvE) while those formed from DHA are termed either resolvins or protectins (or neuroprotectins) (PD) of the D series (RvD) or Maresins (MaR) (Bannenberg et al., 2005, Serhan et al., 2000).

Figure 1.12 shows the biosynthetic pathways involved in the conversion of EPA to RvE1 and RvE2. Via the action of acetylated COX-2, EPA is oxygenated at the C18 position to form 18R-HEPE. By the action of 5-LOX, 18R-HEPE is oxygenated to form 5S-hydro (peroxy)-18RHEPE. The 5S-hydroperoxy group in 5S-hydro (peroxy)-18RHEPE is converted to a 5,6-epoxide intermediate to form RvE1or is reduced to a 5S-hydroxyl group to form RvE2 (Serhan et al., 2002, Hong et al., 2003). Resolvins act to reduce the cellular inflammation in many ways, including: transporting chemicals and inflammatory cells to the sites of inflammation, inhibiting the inflammatory production, and stimulating the expression of molecules involved in anti-inflammatory actions (Dona et al., 2008, Serhan, 2007).

DHA is converted into D-series resolvins. In the absence of aspirin, COX-2 converts DHA to 13*S*-hydroxy-DHA (13S-HDHA) while 15-LOX converts DHA to 17S-hydroxy-DHA (17*S*-HDHA). 17*S*-HDHA is the precursor of four types of D-series resolvins: RvD1, RvD2, RvD3 and RvD4. In the presence of aspirin,

17(*R*)-hydroxy docosahexaenoic acid (17*R*-HDHA) is generated from DHA by the action of aspirin-acetylated cyclooxygenase-2. 17*R*-HDHA is further metabolised by 5-LOX to produce 17-epi-RvD1 (17R-RvD1), also known as aspirin-triggered (AT) RvD1 (Serhan et al., 2002, Hong et al., 2003, Sun et al., 2007) (Figure 1.13).

Two receptors are known to be involved in the action of RvE1: G-protein-coupled receptors GPCR chemokine-like receptor 1 (CMKLR1 or ChemR23) (Arita et al., 2005) and the leukotriene B4 receptor (BLT1) (Arita et al., 2007).

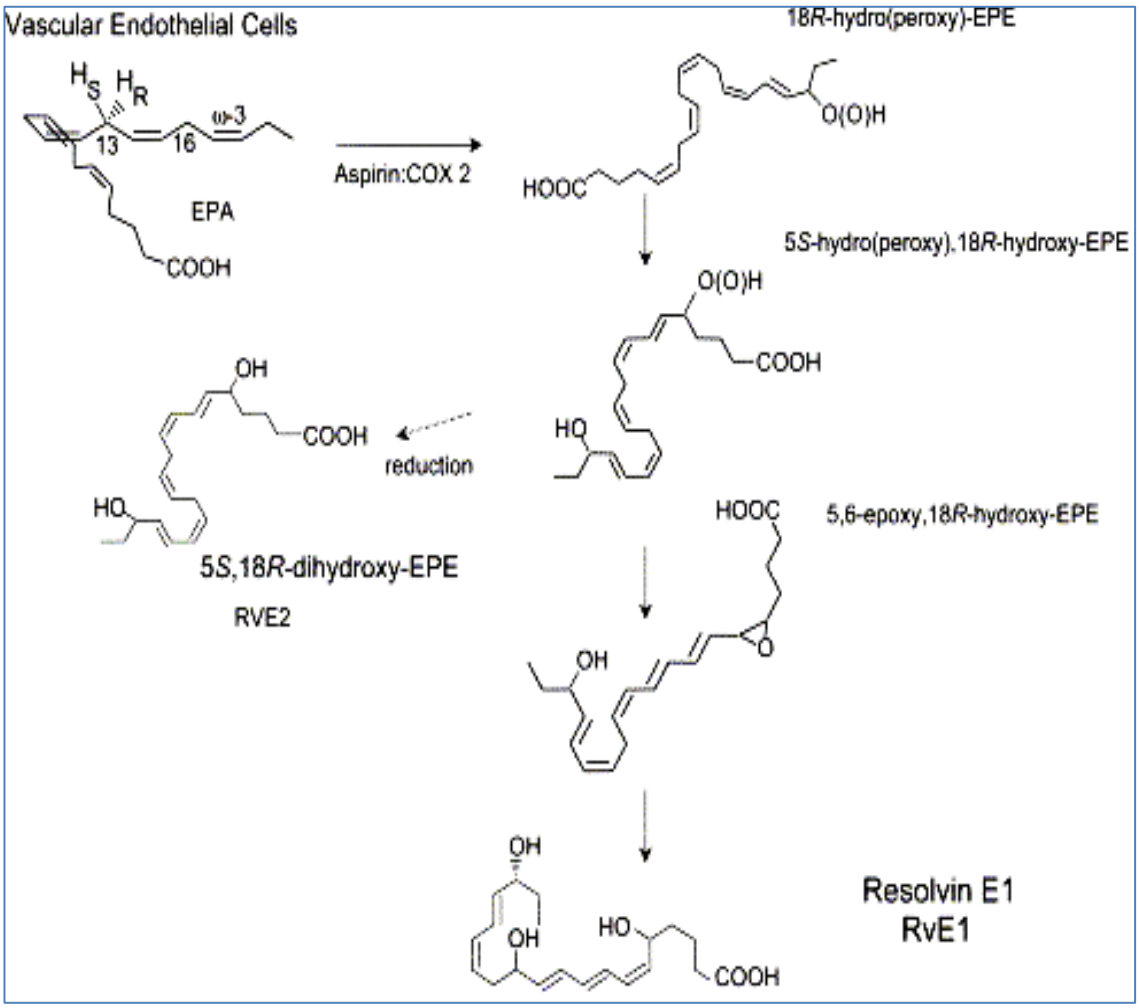


Figure.1.12 Pathways for biosynthesis of RvE from EPA (taken from Serhan et al., 2002)

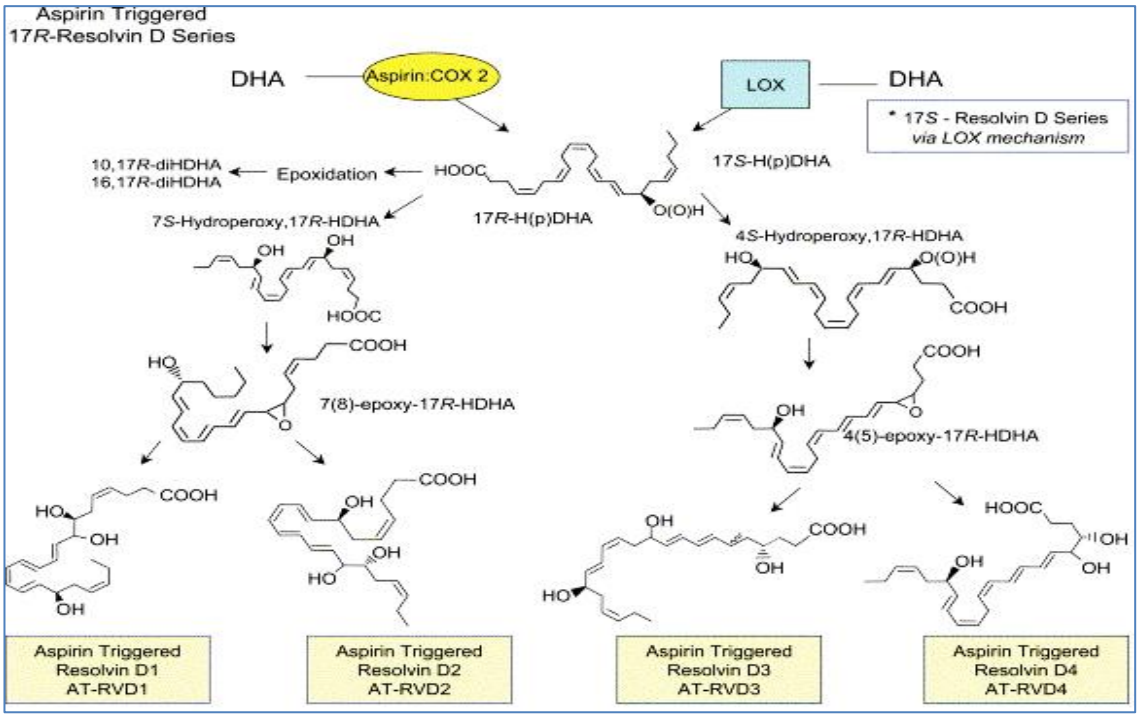


Figure.1.13 Pathways for biosynthesis of RvD from DHA (taken from Serhan et al., 2002).

Protectins (Neuroprotectins) (PD) were discovered in brain tissue as a response to aspirin treatment. 17S-HDHA is converted by the action of LOX to a 16(17)-epoxide and then to the 10,17-dihydroxy docosatriene (10R,17S-DT or PD1 or NPD1). In the early stages of neural diseases and neural injury NPD1 is synthesised and acts as a initial protective signal of cell homeostasis (Bazan, 2013, Zhao et al., 2011) (Figure 1.14).

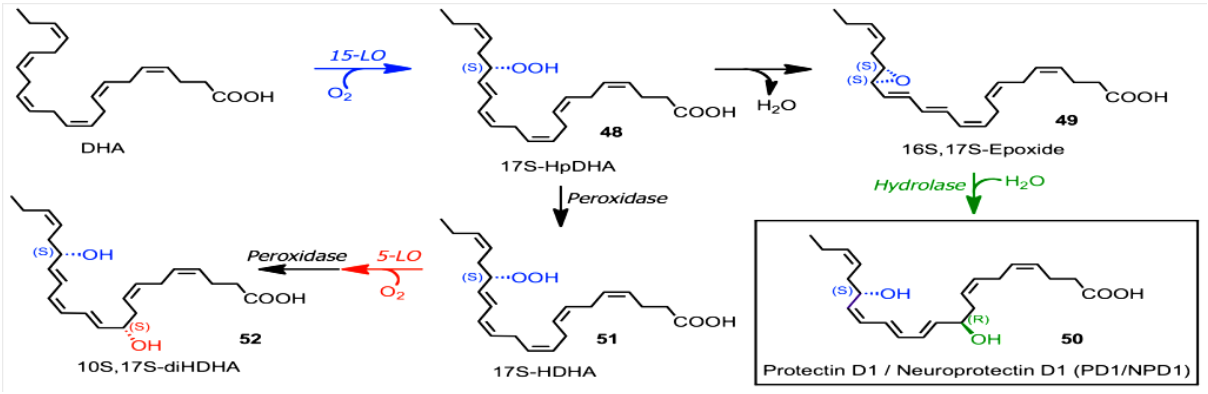


Figure.1.14 Pathways for biosynthesis of PD1 and NDP1 from DHA (taken from Serhan and Petasis, 2011)

Finally, maresins (MaR) are dihydroxy DHA derivatives coined from macrophages. By the action of 12-LOX. DHA is converted to 14-hydroperoxy-docosahexaenoic acid (14S-HDHA) which is reduced to generate 7*S*,14*S*-diHDHA (Serhan et al., 2009). Their biosynthetic pathway is shown in Figure 1.15.

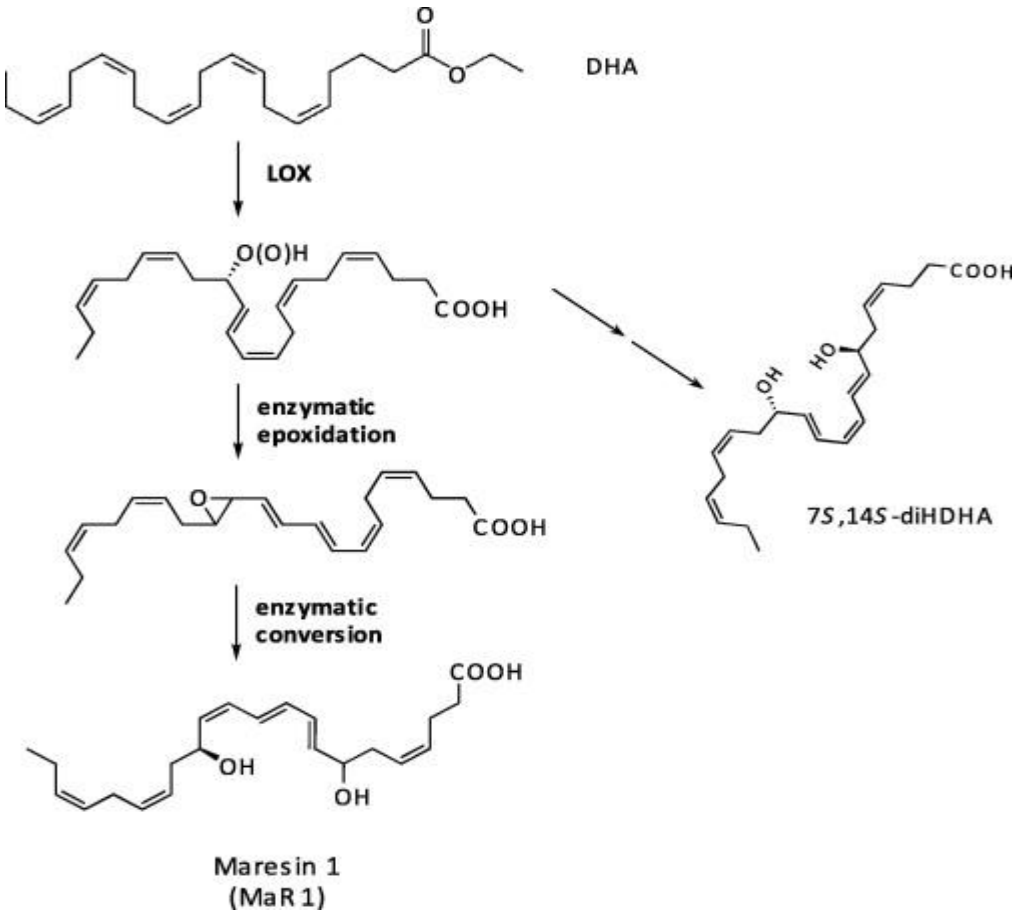


Figure.1.15. Pathways for biosynthesis of maresins from DHA (taken from Stables and Gilroy, 2011)

1.4.5. Cytochrome p450-derived lipid mediators

Cytochrome P450 (CYP) is a family of epoxygenases and monooxygenases that can oxidise PUFA to produce many types of eicosanoids, including epoxides, midchain hydroxy fatty acids and dihydroxy fatty acids. CYP can oxidise AA to produce epoxyeicosatrienoic acids (EETs) and oxidation can

happen at any of the four double bonds of AA, giving 5,6-, 8,9-, 11,12- and 14,15-EET (Campbell and Harder, 1999, Spector et al., 2004). By the action of soluble epoxide hydrolase (sEH) EETs are converted to dihydroxyeicosatrienoic acids (DHETs) (Capdevila et al., 1988, Capdevila et al., 2000). EET species are found in heart, liver, kidney and plasma and include the AA species 8,9-, 11,12- ,14,15-EET (Karara et al., 1992) (Figure 1.16). Epoxyeicosatrienoic acids function primarily as autocrine and paracrine mediators in the cardiovascular and renal systems and also have anti-inflammatory effects in the vasculature and kidney. Many potentially beneficial actions of EETs are attenuated upon conversion to DHETs, which are not essential under normal conditions (Spector and Norris, 2007). Furthermore, EETs have been found to increase angiogenesis and tumour progression, while DHETs inhibit angiogenesis, tumour progression and metastasis (Zhang et al., 2013). Monooxygenases convert AA to hydroxyeicosatetraenoic acids such as 20-hydroxyeicosatetr-aenoic acid (20-HETE). 20-HETE is a pro-inflammatory mediator that stimulates production of cytokines and chemokines such as IL-8, IL-13 and PGE_2 (Ishizuka et al., 2008). 20-HETE can also stimulate endothelial cell proliferation and migration. In the circulation, 20-HETE acts as a vasoconstrictor (Miyata and Roman, 2005).

EPA and DHA are also metabolised by CYP450 in human and animal tissues. CYP450 convert EPA to give: epoxyeicosatetraenoic acids (EEQs) and 8,9-, 11,12- 14,15-, 17,18-DHET and mono- hydroxyeicosapentaenoic acids (20- and 19-HEPE) whereas CYP450 convert DHA to epoxydocosapentaenoic acids (EDPs) 7,9- ,10,1- ,13,14- ,16,17-and 19,20- EDP and mono-hydroxydocosa-hexaenoic acids (22- and 21-HDoHE) (Van Rollins et al., 1988).

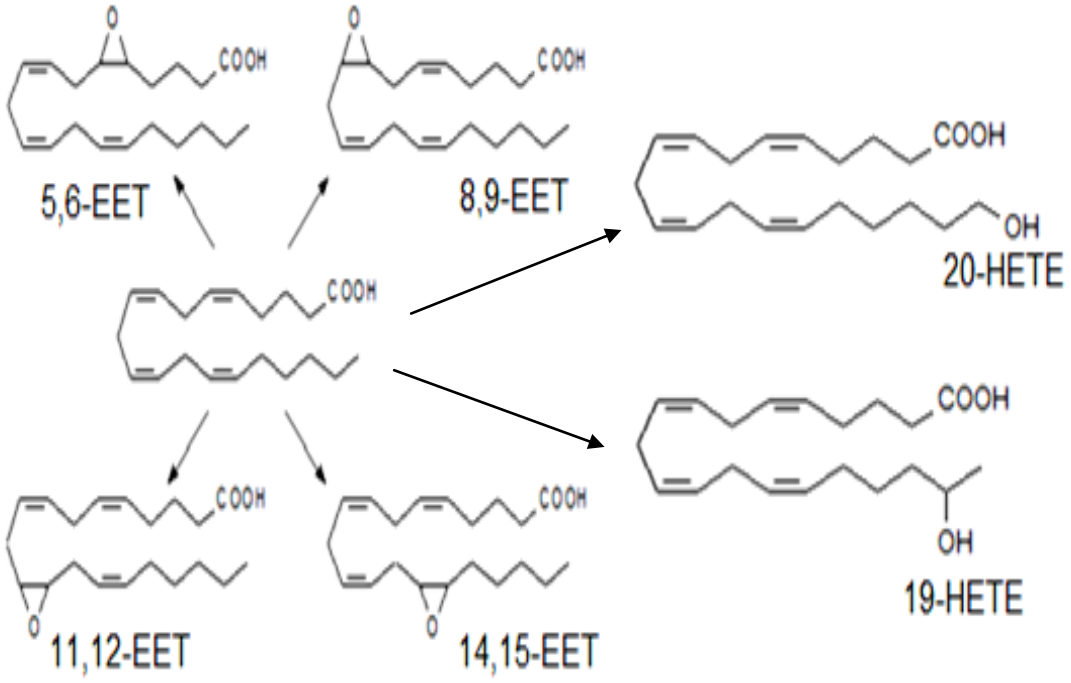


Figure.1.16 Metabolism of arachidonic acid by cytochrome P450 oxygenases (taken from Vriens et al., 2005).

1.5. Eicosanoids in skin inflammation

The skin, specifically the epidermis is a very active site of lipid synthesis in the body (Feingold, 1991). Keratinocytes are able to synthesise most of the fatty acids and this may increase during normal epidermal differentiation. FAs are found esterified in triglycerides, phospholipids, glycosyl-ceramides and ceramides, which have multiple roles in the epidermis such as formation of the epidermal permeability barrier (Uchida and Holleran, 2008). They also function as molecules for energy generation and storage as well as potent signalling molecules that can activate the nuclear hormone receptors (Schmuth et al., 2007) and peroxisome proliferators-activated receptors (Sertznig et al., 2008). Skin lipids have many biological roles ranging from structural to protective (Kendall and Nicolaou, 2013).

Eicosanoids have pro-inflammatory and immunoregulatory activities in human skin. They also play a role in the regulation and differentiation of the epidermis. PGs specifically PGE_1, PGE_2 and PGD_2 have been shown to boost keratinocytes proliferation (Pentland and Needleman, 1986). In contrast, fibroblasts are less responsive to PG pathway activators, whereas keratinocytes can activate fibroblasts to produce prostacyclin which is a paracrine prodifferentiating agent for keratinocytes (Baden et al., 1992).

In human keratinocytes, PGE_2 and $PGF_{2\alpha}$ are the major PGs and their concentrations are increased after UVB exposure (Black et al., 1981, Storey et al., 2007). PGE_2 is the main prostaglandin identified following UVB exposure in cultured human epidermal keratinocytes (Miller et al., 1994). PGD_2 has been found to be a major PG in epidermal melanocytes and FM55 melanoma cells. The regulation of PGD_2 in FM55 occurs by the action of α-melanocyte-stimulating hormone and is not related to melanin production (Masoodi et al., 2010). It has also been reported that PGD_2 can inhibit the growth of human melanoma cells (Bhuyan et al., 1986) and the ability of Langerhans cells to migrate (Angeli et al., 2001).

DGLA, directly generated from dietary arachidonic acid, is normally found in small amounts in the human epidermis. GLA is also converted to DGLA in the human epidermis by the action of an elongase (Huang and Ziboh, 2001). It is metabolised by epidermal cyclooxygenase to series 1 prostaglandins, PGE1 and also by 15-lipoxygenase to 15-HETrE (Miller and Ziboh, 1988).

12-HETE was first reported in human skin in 1975 (Hammarstrom et al., 1975). 12-HETE has been reported to be a major AA product in normal human skin (Nugteren and Kivits, 1987). Moreover, it is mainly formed in the upper

layer of skin epidermis (Henneicke-von Zepelin et al., 1991). The level of 12-HETE increased in cutaneous inflammatory (Black et al., 1985) and post UVR exposure (Grundmann et al., 2004, Rhodes et al., 2009).

It has been reported that LA is the main PUFA in human skin and it has a role in the maintenance of the epidermal water barrier. By the action of 15-LOX, LA is converted to 13-HODE which is then incorporated into the epidermal ceramides and phospholipids (Cho and Ziboh, 1994). It has also been reported that expressions of 5-LOX and LTB_4 concentration were significantly higher in skin suction of systemic sclerosis patients compared with the control. Furthermore, LTB_4 has been found to be higher in cultures of human fibroblast patients (systemic sclerosis) compared with the control (Kowal-Bielecka et al., 2001).

1.5.1. Eicosanoids and atopic dermatitis

Atopic dermatitis (AD) is identified as a chronic allergic inflammatory disease and is characterised by abnormal skin barrier formation (Oyoshi et al., 2009). Epidermal keratinocytes play a role in atopic dermatitis by their ability to both produce inflammatory mediators and to respond to inflammatory and allergenic stimuli (Albanesi, 2010). Atopic dermatitis is characterised by increased activity of PLA_2 (Schafer and Kragballe, 1991). PGD_2 is involved in AD as a result of activating Langerhans cells and mast cells which are the main cutaneous producers of PGD_2 (Matsushima et al., 2011). Although 5-LOX and LTB_4 have been shown to be involved in AD in animal models LOX-derived mediators are less involved in AD compared to COX-derived mediators (Honda et al., 2010). PGI2 is considered vasoactive while, PGE_2 is immunosuppressive and LTB_4 may be involved in the biochemical processes leading to atopic

dermatitis (Fogh et al., 1989, Nakajima et al., 2010). Finally, PUFAs such as EPA, DHA and GLA may be able to improve atopic dermatitis. PUFAs incorporated in membrane phospholipid can change the profile of eicosanoids resulting in a modified function of inflammatory cells (Gueck et al., 2004).

1.5.2. Eicosanoids and psoriasis

Psoriasis is a chronic inflammatory and proliferative skin disease, with genetic and environmental aetiologies (Bhalerao and Bowcock, 1998). In general, psoriasis is characterised by abnormalities in skin lipids, increased production of inflammatory mediators such as IFN-γ, TNF-α and IL-12, and excessive growth and aberrant differentiation of keratinocytes (Lowes et al., 2007, Pietrzak et al., 2010). Psoriasis is also associated with multi organ complications and abnormalities such as increased risk of cardiovascular abnormalities, dyslipidemia, atherosclerosis, diabetes mellitus type 2, obesity, cerebral stroke, cancer, osteoporosis, and depression (Pietrzak et al., 1998, Nijsten and Wakkee, 2009).

It has been reported that levels of free AA and 12-HETE are increased in psoriasis. In contrast, the increase in PGE_2 and $PGF_{2\alpha}$ levels is less prominent (Grabbe et al., 1984). Increases in sPLA2, cPLA2 and pityriasis lichenoides chronica (PLC) activity in psoriatic skin have been shown by many studies, which may explain the increases in AA (Andersen et al., 1994, Cunningham et al., 1985, Sjursen et al., 2000). Furthermore, PGE_2 and $PGF_{2\alpha}$ are low compared with free AA, possibly due to the presence of a natural inhibitor of COX in psoriatic skin (Ikai, 1999). AA can be metabolised by other pathways such as the 5- and 12-LOX pathways. No increase in 5-LOX production is found in peripheral polymorphonuclear leukocytes and platelets in psoriasis (Vila et al.,

1990, Sola et al., 1991). However, the low 5-LOX activity in epidermal keratinocytes suggests that 5-LOX products may be formed through transcellular pathways involving dermally infiltrating neutrophils (Fordhutchinson, 1993).

12-HETE is the predominant eicosanoid found in psoriasis and it is raised due to increased AA production and not because of 12-LOX activation. 12-LOX activity in uninvolved psoriatic skin was no higher than in normal skin. Specific inhibitors for 12-LOX and PLA_2 showed improvement in psoriasis treatment (Burke, 2001). 15-LOX can produce many anti-inflammatory mediators, and 15-HETE is the predominant product in normal dermis. 15-HETE can inhibit 5- and 12-LOX product formation (Kragballe and Voorhees, 1987), counteracting the chemotactic potency of 12-HETE and LTB4 (Kragballe et al., 1986) and acting as a substrate for the formation of lipoxins (Serhan et al., 2003). Supplementation with n-3 PUFA specifically EPA can reduce AA-derived HETE and increase the concentration of anti-inflammatory n-3 PUFA derivatives such as 15-HEPE and 15-HETrE (Mayser et al., 2002, Ricketts et al., 2010, Burke, 2001).

1.5.3. Eicosanoids and sunburn

Sunburn is a skin response to UVR which is the major environmental factor contributing to skin damage. Sunburn is characterised clinicallyby erythema and histologically by dermal infiltration of neutrophils and lymphocytes, and keratinocyte apoptosis. Some of the soluble mediators contributing to these changes include nitric oxide (NO) and cytokines (Rhodes et al., 2001, Rhodes et al., 2009). UVR, specifically UVB has been found to increase COX and LOX mediators in the skin. Through the generation of ROS, UVR can activate gene

expression, transcription factors and signalling cascades in skin cells (Hruza and Pentland, 1993). UVR can also stimulate the activation and expression of $cPLA_2$ in keratinocytes (in vitro), and in human and animal skin (Chen et al., 1996). Studies in animal skin cells reported that mRNA expression of both prostaglandin synthases and receptors are increased after exposure to UVR (Carsberg et al., 1995). Many eicosanoids including PGE_2, 12-HETE, 15-HETE and 13-HODE have been found to be upregulated by UVR (Honda et al., 2009, Rhodes et al., 2009, Nicolaou et al., 2012). Moreover, work with human skin sections and keratinocytes showed that 15-HETE produced by the dermis layer can inhibit 12-HETE in the epidermis layer (Yoo et al., 2008). Furthermore, PGE_2 can arbitrate the primary inflammatory phase and may later take part in the reduction of inflammation and the tissue repair stage. 12-HETE can also be involved in keratinocyte and fibroblast mediated healing events, whilst 15-HETE and 13-HODE may assist in restricting pro-inflammatory signals (Pentland and Needleman, 1986, Ruzicka, 1992).

1.6. Aims and objectives of the project

The main function of the skin is to protect the body against harmful factors such as chemicals, bacteria, UVR and pollution. UVR is the most important factor in the development of erythema, DNA damage, immunesuppression and skin cancer. The toxic effects of UVR from natural sources and artificial lamps are concern for human health. UVR affects lipid metabolising enzymes, modulates their expression and stimulates the production of bioactive lipids by skin cells (Takashima and Bergstresser, 1996, Yoo et al., 2008). Lipid mediators are derived from cellular membranes as a result of activation of membrane phospholipase A_2 by UVB in particular the n-6 PUFA AA and n-3 PUFA EPA (Rhodes et al., 1994). Skin has biochemical and immunological features that can protect the body against UVR. Diet has been recognised as a safe method of protecting the skin and creating an anti-inflammatory environment. n-3 PUFA has been suggested as having a beneficial role in skin health and disease (Sies and Stahl, 2004).

The hypothesis is that n-3 PUFA especially EPA and DHA can protect skin cells against UVR through the formation of novel lipid mediators. Therefore, the purpose of this study was to measure and evaluate this protective effect using a model system comprising HaCaT keratinocytes and 46BR.1N fibroblastic cells. The cells were treated with fatty acids and UVR. Furthermore, samples from a clinical study were used in order to assess the effect of oral n-3 PUFA supplementation on skin fatty acids. The subjects were supplemented with n-3 PUFA for 3 months.

The specific objectives of this project were as follows:

A) To investigate the effect of UVR dose on cell viability and establish a dose suitable for the in vitro study.

B) To assess the toxicity of n-3 PUFA on the cell lines used in this study and decide on a suitable dose for the in vitro treatments.

C) To assess the effect of UVR or/and n-3 PUFA on HaCaT and 46BR.1N cell apoptosis.

D) To assess the effect of n-3 PUFA on the fatty acid profile of the cell lines used in the study.

E) To measure the eicosanoids produced by HaCaT and 46BR.1N cells and the effect of UVR and n-3PUFA on them.

F) To assess the systemic effect of n-3 PUFA on skin lipids using red blood cells and skin samples through supplementation in a clinical study.

Chapter 2. Materials and Methods

2.1. Cells and clinical samples

2.1.1. Cell lines

The HaCaT human keratinocyte cell line was provided by Dr S. Britland of the University of Bradford. The 46BR.1N human fibroblast cell line was purchased from The European Collection of Cell Culture (ECACC).

2.1.2. Clinical samples

2.1.2.1 Participants

All aspects of the work described in section 2.1.2 were undertaken by Prof L.E Rhodes and her group at Manchester University. Seventy nine (79) healthy female volunteers with nickel allergy were recruited for the study. Volunteers were eligible to take part if they were aged between 18-60 years and of sun reactive skin type I or II. Volunteers were excluded from the study if they were taking n-3 PUFA supplements, were pregnant or breast feeding, had sunbathed or used a tanning bed in the three months prior to the study, were taking photoactive medication, or had a history of photosensitivity, skin cancer or atopy. Ethical approval was granted by North Manchester Local Research Ethics Committee (08/H1006/30) and the study was performed in accordance with the Declaration of Helsinki principles. Written informed consent was provided by all volunteers before their inclusion in the study. Baseline characteristics of the women recruited for this study are presented in Table2.1.

2.1.2.2. Study Design and Supplements

The subjects were computer-randomised by the study's biostatistician into the two treatment groups: Group A (active group, n= 40 subjects) was supplemented with n-3 PUFA. The n-3 PUFA supplements were 1g gelatine

capsules containing Incromega E7010 SR ethyl ester which comprised ~72% EPA, 10% DHA. Meanwhile, Group B (placebo group, n=39 subjects) was supplemented with placebo supplements. The placebo supplements comprised 1g gelatine capsules of identical appearance containing glyceryl tricoprylate coprate (GTCC). Volunteers were given five capsules of either n-3 PUFA or placebo supplements per day with breakfast for 12 weeks. The studies were performed at the end of the 12-week period. Seven ml of blood was taken (pre- and post-supplementation) from each volunteer for assessment of red blood cell (RBC) fatty acids content. The samples were collected in 3.4ml ethylenediamin-etetraacetic acid (EDTA) monovette tubes. Samples were then centrifuged for 15 minutes at 1500rpm and the RBC fractions were collected and stored at -70ºC. Five mm diameter skin punch biopsies were taken from an unexposed site of upper buttock skin under local anaesthesia and placed into 1.8mL cyrotubes before being snap froze in liquid nitrogen and stored at -70ºC. The red blood cells and skin punch biopsies were pre- and post-supplementation. The samples transported in dry ice and were stored at -80ºC until analysis.

Table 2.1. Baseline characteristics of women recruited in the clinical study

Demographic	**Range**	**Mean ± SD**
Age (Years)	21-60	42.8±10
Body mass index (kg/m^2)	19.4-44.2	26.7±5
Smoking status*	**Number of volunteers**	**% of total volunteers**
Never smoked	30	39
Ex smoker	18	27
Current smoker	13	17

* Missing data n=17 (22%)

2.2. Cell culture

2.2.1. Materials

Hank's Balanced Salt Solution (HBSS) was purchased from Promo Cell Gimbh, Heidelberg, Germany. Dulbecco's Modified Eagle Medium (DMEM) was purchased from Invitrogen, UK. RPMI 1640 media, Minimum Essential Medium Eagle (MEME) media, fetal calf serum heat inactive (FCS), fetal bovine serum (FBS), non-essential amino acid solution, dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), sodium pyruvate, oleic acid (OA; 99% purity), cis-5,8,11,14,17-eicosa-pentaenoic acid (EPA; 99% purity), cis-4,7,10,13,16,19-docosahexaenoic acid (DHA; 98% purity), penicillin (10,000U/ml) streptomycin (10mg/ml), amphotericin B (2.5ng/ml), 0.4% trypan blue solution and 0.25% trypsin-EDTA (1mM) solution were all purchased from Sigma, UK. 15ml centrifugation plastic tubes, 50 ml self standing centrifugation plastic tubes, T-25, and T-75 culture flasks, 6, 12, and 24 well cell culture cluster and 96-well microplates were all purchased from Corning, Amsterdam, Netherlands. 25ml plastic tubes, tissue culture dishes (100/20mm) and serological plastic pipettes 10ml were purchased from Sarstedt, Leicester, UK. Ethylenediaminetetraacetic acid (EDTA) monovette tubes were purchased from Sarstedt, Newton, UK. APOPercentage kit was purchased from Biocolor Life Science Assay, Northern Ireland, UK.

2.2.2. Equipment

Microscope: CK40-F200, Olympus, Japan. UV lamp: UV 236 B, Waldmann, Germany. Light meter; Waldmann, Germany. Centrifuge: Rolofix 32 Ilettich entrifugen, Scientific Laboratory Supplies, Germany. Shaker: MS1 Minishaker, IKA-Labortachnik, Brasil. Incubator: Heraeus CO^2 incubator BBD

622, Thermo Scientific, UK. Microplate reader: ELx800, BioTek Instruments, USA. Aspirator: Vacumsafe confort, IBS Integra Biosciences, Switzerland. Thermocouple, UK and Improved Neubauer haemocytometer, Gallencamp, UK.

2.2.3. Methods

2.2.3.1. Cell culture and passaging

HaCaT cells were grown in complete RPMI 1640 media (Appendix1.1) or complete DMEM media (Appendix 1.1) depending on the experiment. 46BR.1N cells were grown in complete MEME media (Appendix 1.1). All cells were grown in T-75 culture flasks at 37°C, 5% CO_2 in an incubator. The cells were observed every day under the microscope to ensure that they were continuing to grow. When the cells reached about 90% confluences they were passaged. All cells were cultured up to a 10^{th} passage per experiment. All equipment was sprayed with 70 % ethanol before being placed in the flow hood. The media that covered the cells were removed and discarded into a waste beaker containing disinfectant. Then, 5ml of pre-warmed (37°C) HBSS solution was added to each T-75 flask. In order to wash the cells, the HBSS solution was gently swirled inside the flasks and then discarded. 8 ml of warm (37°C) 0.25% trypsin-EDTA solution was then added to each T-75 flask to detach the cells. The flasks were placed back in the incubator for 15 min. Then, the flasks were held up to the light to check that all cells were detached. The cells were also checked under the microscope to ensure that they were completely detached. When it was confirmed that all cells were floating, trypsin was neutralised by adding 4ml of the appropriate complete cell culture medium to the T-75 flask and the contents of the flask were transferred to a 15 ml centrifuge tube. The tube was spun down (2,000 rpm, for 5 min), and the supernatant was removed and replaced by

4 ml of fresh complete media. The cells were then resuspended by gently pipetting the media up and down. Then, the cells were counted and an appropriate volume of cell suspension was transferred into new cell culture flasks or cell culture plates, as needed. Fresh medium was added to cover the surface of the flasks (8 ml toT-75 and 10 ml to100/20 mm Petri dishes). All flasks and/or plates were then placed in the incubator and used for further work.

2.2.3.2. Cell counting

In order for the cells to be counted, 100µl of the original cell suspension was transferred to an Eppendorf tube. A dilution of 1:1 was conducted when the cell suspension was mixed with 100µl of 0.4% trypan blue solution. A Neubauer haemocytometric slide with coverslip was prepared and 10µl of this cell suspension was transferred to the chamber. The cells were counted in 4 grids per chamber under the microscope, using the 10x focus. The average number of cells of 4 grids was calculated and this number was used to count the total number of cells by using the following calculations:-

Number of cells/ml = [average cell number] x [10^4] x [dilution factor (2)]

Total number of cells = [Cells/ml] x [volume of original cell suspension (ml)]

2.2.3.3. Cell treatment with Ultraviolet Radiation

2.2.3.3.1. Effect of UVR exposure on temperature in the UV box

Prior to exposing the cells to UV light, it was necessary to measure the temperature inside the UV box to ensure that it was stable and did not increase during the UV exposure and affect cell viability. A thermocouple was used to measure the temperature in the UV box. The thermocouple was put in a Petri dish containing 5ml of PBS. The temperature was tested before and after the

UV lamp was turned on for various lengths of time corresponding to 40, 80, 120, 160, 180 and 220 mJ/cm^2.

2.2.3.3.2 Exposure of cells to UV radiation

When cells reached about 80% confluence (12-well cell culture plates or cell culture dishes, depending on the experiment) the medium was removed and the cells were washed with 0.5 ml/well or 5 ml/dish pre-warmed (37°C) PBS to remove any remaining media and floating cells. Cells were then covered with a small volume (500µl/well or 5ml/dish) of pre-warmed PBS and exposed to UVR. The UV lamp was switched on and left for 10 min to stabilise. A light meter was used to measure the irradiance (mW/cm^2) of the lamp to ensure that the lamp had stabilised. Typically, this value was 0.33 mW/cm^2. The cells were then placed at the base of the UV box, 30 cm under the UV lamp and irradiated for the required amount of time. The exposure time was calculated as follows: Exposure time (Sec) = Dose (mJ/cm^2)/UVR intensity (mW/cm^2). This corresponded approximately to 45 sec for 15mJ/cm^2. After that, the PBS was removed from the wells or dishes and replaced with the appropriate amount of fresh media depending on the experiment. The plates or dishes were then incubated for the required amount of time.

2.2.3.4. Treatment of cells with fatty acids

All fatty acids (EPA, DHA and OA) were dissolved in DMSO to prepare stock solutions (50 mM), (Appendix 1.1). DMSO is a widely used solvent, it is protects the cell from damage during freezing. DMSO is a good solvent of fatty acid and they are high solubility in it. These stock solutions were further diluted with culture media to give a final concentration of 10 or 50 µM. When the cells reached about 70% confluence, the medium was removed and the cells were

washed with pre-warmed HBSS. The HBSS was replaced by the required amount of fresh complete medium containing the fatty acid treatment: 10 µM fatty acid was prepared by adding 2µl of fatty acid stock solutions (50 mM) and 8µl of DMSO into 10ml of complete medium; 50 µM fatty acid was prepared by adding 10µl of fatty acid stock solutions (50 mM) into 10ml of complete medium or vehicle control DMSO (10 µl) into 10ml of medium. Cell cultures were then incubated for 72-h before being exposed to UV irradiation as described above.

2.2.3.5. Collection of cells and media for various analyses

In order to investigate eicosanoid production, protein expression and cellular fatty acid content, the cells were grown in cell culture dishes diameter 100 and were treated with fatty acids for 72h. After that, the media were removed and the dishes washed with pre-warmed PBS. 5ml of PBS was then added to each dish. The cells were then exposed to UVR as in section 2.2.3.3.2. The control experiments were placed in the UV box for the corresponding time but were not irradiated. PBS was then removed from all dishes and replaced with 10 ml of pre-warmed fresh free serum medium (see Appendix1.1). The plates were then incubated for a further 24h. The medium was collected 24h post UVR by a serological pipette and transferred into a pre-labelled 25ml tube. The medium was stored at -80°C to be used for the analysis of eicosanoids. In order for the cells to be collected 5ml of warm HBSS solution was added to each dish, gently swirled inside the dish and discarded. 8ml of warm (37°C) 0.25% trypsin-EDTA solution was then added to each dish to detach the cells. The dish was placed back into the incubator for 15 min. Then, the cells were checked under the microscope to ensure that they were completely detached. Trypsin was neutralised by adding 3 ml of medium, and the contents of the dish

were transferred to a pre-labelled 15 ml centrifuge tube. The tube was spun down using 2,000 rpm, for 5 min. The supernatant was removed and replaced by 5ml of PBS. The cells were then re-suspended by gently pipetting the PBS up and down, and counted as described in section 2.2.3.2. In order for the cells to be collected and stored, the cell suspension was spun down using 2,000 rpm, for 5 min. Then the supernatant was removed and the cell pellet was stored at -80°C to be used for western plot analyses and fatty acid analysis.

2.2.3.6. Isolation of the epidermal layer from the human skin

Skin was provided by the Ethical Tissue Bank at the University of Bradford. In order to separate the epidermis, the fat was removed from the skin, the skin was washed with PBS and put into a clean 50 ml tube. The tube was then filled with 1M sodium bromide (NaBr; 5.1g in 50ml water) and incubated in a water bath at 37 ^{o}C for 1 hr. After this incubation, the epidermal layer was separated from the dermis using fine forceps. The epidermis was placed in a clean dish topped up by PBS and stored at 4^{o}C until required for use.

2.2.3.7. Effect of placing a skin epidermis layer in the path of UV light

In order to assess the effect of placing a skin epidermis layer in the path of UV light, the irradiance (mW/cm^2) of the UV lamp was measured by a light meter for 10 min. Then, a quartz glass was placed on the light meter and the irradiance was measured for another 10min. Finally, the wet epidermis was placed on the quartz slide this was placed on the light meter and the intensity was measured for 10 min.

2.2.3.8. MTT assay

The MTT assay is a colorimetric method used to determine cell viability. The dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (yellow) was used in this assay. To assess cell viability post UVR and fatty acid treatments, the cells were cultured in 12-well cell culture plates, treated with vehicle (DMSO) and/or fatty acids and exposed to UVR. After the required amount of time post UVR (4h or 24h) the PBS was removed. Then, 1 ml of MTT solution (prepared by mixing 1ml of MTT (5mg/ml) with 9 ml of HBSS) was added to each well. Next, the plate was incubated for 4 h at 37°C. During the incubation, the yellow colour was reduced by enzymes in living cells to give a water-insoluble, purple formazan salt. At the end of the incubation time, the MTT reagent was aspirated and replaced by DMSO (1ml/well) to solubilise the formazan salt. The plate was then put on the shaker for 10 to 15 mins. 200µl solution from each well was transferred into a 96 well microplate, in triplicate. Absorbance of the purple solution was measured at 595 nm using a microplate reader. This is indicative of cell viability, which can be calculated as follows:

Cell viability (%) = (Absorbance of sample/Absorbance of control) x 100

2.2.3.9. Apoptosis assay

In order to assess the effect of n-3 PUFA on cell apoptosis, the cells were cultured in T-75 flasks and treated with DMSO and n-3 PUFA for 48h as described in section 2.2.3.4. Then the cells were seeded in a 24-well cell culture plate as$10x10^4$ cells/well 500µl media and treated with n-3 PUFA for 24h. At 24h post UVR the apoptosis was measured using APOPercentage™ kit. As a result of apoptosis, the DNA in the cells becomes fragmented and phosphatidyl serine is transferred to the outside of the cell membrane. The dye bands to the

phosphatidyl serine and inter in the cell. By the action of Dye release reagent the dye in labelled cells is released into purple-red solution which can measured using a microplate colorimeter.

As per the APOPercentage™ assay protocol, 10mM H_2O_2 (see Appendix 1.1) was added to the positive control samples 4h before the incubation time ended. Nothing was added to the reagent blank sample. The medium was removed 30 min before the incubation time end and replaced with reagent A (see Appendix 1.1). After 30 min the medium was removed and replaced with reagent B (see Appendix 1.1). Plates were incubated for 30 min at 37°C/under 5%CO_2. The medium was removed from the wells and gently washed twice with 500µl/well PBS. 50µl of trypsin was added to each well and the plates were incubated for 10 min at 37°C/5%CO_2. 200 µl of dye release reagent was added to each well. The plates were put on the shaker for 10 min. The contents of each well (200 µl) were transferred into 96-well microplates and the absorbance of the purple-red solution was measured at 550nm. This is indicative of cell apoptosis, which can be calculated as follows: Cell apoptosis (%) = (absorbance of sample - absorbance of reagent blank sample) / absorbance of control x 100

2. 3. Fatty acid analysis

2.3.1. Materials

Potassium carbonate (ACS reagent), 2,6-di-tert-butyl-4-methylphenol (BHT) (ACS reagent), sodium sulphate (anhydrous) (ACS reagent), 14% boron trifloride in methanol (BF_3) and potassium chloride (ACS reagent) were purchased from Sigma Aldrich, UK. Trimethylpentane and toluene were

purchased from ACROS organics, Loughborough, UK. Methanol (HPLC grade), chloroform (HPLC grade) and dichloromethane (HPLC grade) were all purchased from Fisher Scientific, Loughborough, UK. FAME mixed standard, vaccenic (C18:1 n-7) methyl ester and docosapentaenoic acid methyl ester (C22:5n-6) were purchased from Sigma Aldrich, UK. 50 and 250 µl glass syringes were purchased from SGE, Australia. Glass tubes and glass Pasteur pipettes were purchased from Fisher, Loughborough, UK. Amber glass vial, 100 µl insert vials, screw caps, and septa were all purchased from Kinesis, Bedfordshire, UK.

2.3.2. Equipment

GC-FID: 6850 Series and autosampler: 6850 gas chromatography systems and GC operated system: Chemstation Revision B2.01software, Agilent Technologies UK. Hydrogen gas generator: Parker Balston, Parker Hannifin, England. Column: BPX-70, 0.25mm ID 0.25µm film 60mL; Phenomenex, UK. Vortex: Whirlmixer, Flsons Scientific Apparatus, England. Centrifuge: Sorvall RT6000B refrigerated centrifuge, Dupont, LTD (UK), Stevenage, Herts. Heating block: Reacti-Therm heating module, Pierce, UK.

2.3.3. Methods

2.3.3.1. Preparation of samples

A) The red blood cell samples were defrosted on ice and brought to room temperature. RBC (1 ml) was transferred by Pasteur pipette to a clean pre-labelled extraction tube (T1) which was placed in ice. To ensure that all the blood was transferred, the original vial was washed by 1 ml of water and also transferred to T1. Five ml of ice cold 0.01% (w/v) BHT in 2:1 chloroform:

methanol (see Appendix 1.2) was added to T1. The sample was extracted immediately as described in section 2.3.3.2

B) Frozen dermal skin section: the vials containing the samples were placed on ice to gently defrost. Once thawed, excess blood was removed from the tissue by handling it with tweezers and washed it in a vial containing fresh ice cold PBS. The vial was gently shaken for 30 sec; the biopsy was weighed and transferred to an extraction tube T1 containing 4 ml of ice cold 0.01% (w/v) BHT in 2:1 chloroform: methanol. The tube was sealed with parafilm and was incubated overnight at 4°C. The sample was extracted as described in section 2.3.3.2

C) Frozen cell pellets: the tube containing the cell pellet was placed on ice to gently defrost. Then, 1ml of PBS was added to the cell pellet; the pellet was gently suspended and transferred to a pre-labelled extraction tube T1 containing 4 ml of ice cold 0.01% (w/v) BHT in 2:1 chloroform: methanol. The sample was extracted immediately as described in section 2.3.3.2

D) n-3 PUFA supplements (capsule): In order to assess the purity of the supplement and to confirm the EPA/DHA content of the capsules in the clinical study, their content was analysed for fatty acid ethyl ester (n=3 active and n=3 placebo) and total fatty acids (n=3 active and n=3 placebo) by GC-FID. Specifically:

The capsules were opened using a scalpel and 25µl of the oil was aspirated using a glass syringe. The oil was transferred to aclean volumetric flask and dissolved in 25ml of hexane (stock solution). Then, 140 µl of the stock solution was transferred to a clean glass tube T1. 60 µl of C21:0 (Internal

standard, 900ng/ µl) was then added and the solution thoroughly mixed. The resulting sample solution was then transferred to a clean 100 µl insert using a glass syringe and, placed in an amber glass vial. The sample was immediately analysed for fatty acid ethyl esters. For assessment of fatty acid methyl ester (FAME), another aliquot of this sample solution was derivatised prior to analysis, as described in section 2.3.3.5. All analyses were carried out using GC-FID in split mode as described in section 2.3.3.6.

2.3.3.2. Lipid extraction

The extraction tube T1 was then vortexed for 5 x 1 min. Between vortexing steps, T1 was kept cool on ice for at least 30 sec (1 ml of water was added to the skin extraction tube T1). The samples were centrifuged at 4^{o}C, 5000 rpm for 5 min. The lower layer (organic) of extract was transferred to pre-labelled, clean extraction tube T2 and kept on ice. To the first tube T1, 4ml of 0.01% (w/v) BHT in 2:1 chloroform: methanol was added. T1 was vortexed for 5 x 1 min and centrifuged as above. The lower layer was then transferred and combined with the extract in T2. This procedure was repeated once more. 2 ml of 0.5M KCL in methanol (50:50, v/v) (see Appendix 1.2) was added to the combined lipid extract in T2. As before, the combined lipid extract T2 was vortexed and centrifuged. The top layer (aqueous) was removed and discarded, and another aliquot of 2 ml of 0.5M KCL in methanol (50:50,v/v) was added. T2 was then vortexed and centrifuged, and the bottom layer was transferred into a pre-labelled, clean extraction tube T3. Approximately four spatula tips of dry sodium sulphate were added to T3, gently mixed. The lipid extract was passed and filtered through cotton wool filters (the filters were prepared with packed cotton wool in Pasteur pipettes, and were previously washed with 3 ml of 0.01%

(w/v) BHT in 2:1 chloroform: methanol, and flashed and dried with air). The filtrate was then collected and transferred to an esterification tube E1. In order to collect any remaining lipid extract, 2 ml of 0.01% (w/v) BHT in 2:1 chloroform: methanol was also passed through the filters. The filter washes were collected and added to E1. The lipids extract in E1 was dried under a fine stream of nitrogen (each sample had a needle of the nitrogen drying apparatus directly above it) and reconstituted in 1ml of 0.01% (w/v) BHT in 2:1 chloroform: methanol. The samples were stored at -20°C for up to a week.

2.3.3.3. Preparation of internal standard methyl ester

In order to prepare internal standard stock solution (1mg/ml), 0.025mg of heneicosanoic acid (C21:0) was dissolved in 25ml of 0.01% (w/v) BHT in 2:1 chloroform: methanol and stored at -20ºC. In order to prepare the internal standard methyl ester, 300 µl or 900 µl of stock solution (1mg/ml) was transferred into a clean tube to esterify as described in section 2.3.3.5. The resulting internal standard methyl ester was reconstituted in 1ml DCM to give a final concentration of 300ng/µl or 900ng/µl. 100µl of internal standard methyl ester was then transferred to an injecting vial and the rest was stored at 20°C for a week.

2.3.3.4. Preparation of FAME cocktail for GC analysis

2.3.3.4.1. Preparation of 1µg/µl (w/v) docosapentaenoic acid stock solution (C22:5 n-3)

Stock standard: commercially available DPA methylester as 1mg/m1 solution in ethanol. The stock standard was dried under nitrogen and then reconstituted in 1ml DMC and stored at -20°C.

2.3.3.4.2. Preparation of 200 ng/µl docosapentaenoic acid working solution (C22:5 n-3)

In order to prepare a 200ng/µl working standard, 200µl of stock standard was transferred to an amber glass vial, diluted with 800µl DCM and stored at -20°C.

2.3.3.4.3. Preparation of 1µg/µl(w/v) vaccenic acid stock solution(C18:1n-7)

Stock standard: commercially available vaccenic acid methyl ester as 1mg/m1 solution in ethanol. The stock standard was dried under nitrogen and then reconstituted in 1ml DMC and stored at -20°C.

2.3.3.4.4. Preparation of 200ng/µl vaccenic acid working solution (C18:1n-7)

In order to prepare a 200ng/µl working standard, 200µl of stock standard was transferred to an amber glass vial, diluted with 800µl DCM and stored at -20°C.

2.3.3.4.5. Preparation of FAME cocktail standard for a split method

In order to prepare the FAME cocktail standard for a split method, 20µl of each of the following was transferred to a glass insert: Commercially available FAME standard, docosapentaenoic acid methyl ester (C22:5 n-3; 200ng/µl) and vaccenic acid methyl ester (C 18:1 n-7; 200ng/µl). A final volume was made up to 200µl of DCM. The solution was mixed and stored at -20°C.

2.3.3.4.6. Preparation of FAME cocktail standard for a splitless method

FAME cocktail standard for a splitless method was made by diluting the FAME cocktail standard for a split method using 1:3 dilution. For this, 20µl of

FAME cocktail standard for a split method was transferred to a round bottomed, glass insert, a 40µl DCM were added and the new solution stored at -20°C.

2.3.3.5. Preparation of fatty acid methyl esters

The samples were prepared for analysis by a splitless method (RBC) or split method (skin and cell pellets). The lipid extracts which were in E1 and stored at -20^{o}C, were defrosted. 15µl or 45µl of internal standard (C21) was added to the lipid extract. The lipid extract was dried to remove the solvent. 250µl of toluene: methanol (50/50 v/v) (see Appendix 1.2) and 250 µl of 14% BF_3-methanol solution were added to the dried lipid extract. The esterification tube E1 was flashed under nitrogen, closed with a Teflon cap and gently vortexed. The heater block set was pre-heated to 100 ^{o}C and the fatty acid esterification tubes were transferred onto it and left for 90 min. After 90 min had elapsed the tubes were transferred to an ice bath. After 10 min on ice, 1.5 ml of 10% K_2CO_3 solution (w/v) (see Appendix 1.2) and 2 ml of trimethylpentane were added to each fatty acid esterification tube. The tubes were vortexed and centrifuged at 4ºC, 5000 rpm for 5 min. The upper layer, which contains the FAME, was carefully transferred to a clean tube E2 using a glass Pasteur pipette. The FAME solution was gently dried under nitrogen, reconstituted in 60µl dichloromethane, vortexed and centrifuged for 10 s at 3000rpm to help collect all the drops from the glass walls. The resulting solution was transferred to a clean 100 µl insert vial using a glass syringe, placed in an amber glass vial, and analysed immediately.

2.3.3.6. Gas chromatographic analysis

Fatty acid methyl esters were analysed by GC-FlD. A mixture of hydrogen and air were used as the detector gas, and helium as the carrier gas. The injection temperature was set at 220 ^{o}C and the flame ionisation detector (FID) was at 250 ^{o}C. The injection volume was 1μl and the GC run time was as follows: The oven temperature was set at 70 ^{o}C for 2 min and was then increased to 150 ^{o}C at the rate of 20 ^{o}C /min for 5 min; it was then elevated to 218 ^{o}C at the rate of 2.50 ^{o}C /min. The rate of increase was 0.60 ^{o}C from 218 ^{o}C to 225 ^{o}C. The temperature was set at 225 ^{o}C for 10 min followed by a rise to 230 ^{o}C at the rate of 2.50 ^{o}C /min; this was then maintained for 3 min. The overall run time was 75 min. Dichloromethane (blank) was injected followed by FAME cocktail standard and internal standard methyl ester to establish the retention times. Blanks were injected between the standard and first sample injection and then this every 4 sample injections.

2.3.3.7. Identification and quantitation of fatty acid methyl esters

In order to establish the retention times of each analyte, and allow identification and quantitation of the fatty acid methyl esters, the internal standard methyl ester and a mixed FAME standard cocktail were injected before the samples. All samples were injected in duplicate. To identify the fatty acids, retention times of FAME in the sample were compared to mixed FAME standard cocktail retention times. In order to give relative amounts of each fatty acid, the FAME peak area in each sample was integrated and normalised using the peak area of the internal standard of the run. Data are expressed as % weight of total fatty acid in the sample.

2. 4. Eicosanoid analysis

2.4.1. Materials

Solid phase extraction (SPE) cartridges (C18-E 500mg, 6ml) were purchased from Phenomenex, Macclesfield, UK. Narrow range (2.5-4.5) pH indicator paper strips were purchased from Merck, UK. Methanol (HPLC grade), ethanol (HPLC grade), and hexane (HPLC grade) hydrochloric acid were all purchased from, Fisher scientific, UK. Methyl formate was purchased from Sigma, UK. 50 and 250 µl glass syringes were purchased from SGE, Australia. Glass tubes and glass Pasteur pipettes were purchased from Fisher, Loughborough, UK. Amber glass vials, 100 µl insert vials, crew caps, and septa were all purchased from Kinesis, Bedfordshire, UK.

2.4.2. Equipment

A Waters Alliance 2695 HPLC pump with a Waters 2690 autosampler coupled to an electrospray ionisation (ESI) triple quadruple Quattro Ultimo mass spectrometer and The Mass Lynx^{TM} V 4.0 operating software were purchased from Waters, Elstree, UK. SPE vacuum Manifold was obtained from Phenomenex, Macclesfield, UK. Whirlmixer was purchased from Flsons Scientific Apparatus, England. Sorvall RT6000B refrigerated centrifuge was obtained from Dupont, LTD (UK).

2.4.3. Sample preparation

In order to analyse the eicosanoids in cell culture media, the cell culture media (10ml) from different treatment were defrosted by transferring the samples from -80C storage to the fridge for 2h. After that, the samples were placed on ice. The 50 and 250 glass syringes were washed with ethanol and

the internal standards $PGB_{2\text{-}d4}$ (1ng/µl) and 12 $HETE_{\text{-}d8}$ (1ng/µl) were mixed 5 times. 40 µl from each internal standard was added to each sample before it became fully defrosted. 1.77 ml methanol was added to each 10ml sample to make 15% methanol. The samples were incubated for 15 min in ice. Then samples were centrifuged at 3000 rpm for 8 min to remove the precipitated protein. The SPE cartridges were labelled and attached to a vacuum manifold. The SPE cartridges were preconditioned with 20ml 100% methanol and washed with 20 ml MQ water. The pressure was adjusted so that the solvent was eluted from the ESP cartridges drop-wise. The samples were acidified with 1M HCl (see Appendix 1.3) of approximately 12-15 drops to adjust to pH 3.0. The pH of the samples was measured by a narrow range pH indicator paper. The sample was immediately transferred to the preconditioned SPE cartridges using a glass Pasteur pipette. Pressure was not used in this step to elute the sample. The SPE cartridges were then washed with 20ml 15% methanol (see Appendix 1.3), 20ml water (glassware were washed with hexane) and 10 ml of hexane. The glassware was washed with methyl formate and finally, 12 ml of methyl formate was added to each SPE cartridge to collect the lipid extraction. The organic solvent was evaporated under nitrogen; the residue was dissolved in 100 µl ethanol/MQ water (70/30 v/v). The samples were mixed, vortexed and centrifuged for 1min. The samples were transferred to clean 100 µl insert vials, placed in amber glass vials and stored at -20C for up to one week for analysis.

2.4.4. LC/ESI-MS/MS Analysis

LC/ESI-MS/MS analysis was performed on a Waters Alliance 2695 HPLC pump with a Waters 2690 autosampler coupled to an electrospray ionisation (ESI) triple quadruple Quattro Ultimo mass spectrometer. The Mass Lynx^{TM} V

4.0 was used as operating software to control the instrument and data acquisition. The instrument was operated in the negative ionisation mode. The sample chamber was a Waters 2690 autosampler and the column temperature was set up at 8°C. The source temperature was 120°C and the desolation temperature was 360°C. The capillary voltage was set at 3500V and the cone voltage at 35V. Collision energy settings and multiple reaction monitoring (MRM) transitions for each detectable compound were set up as described by Masoodi and Nicolaou (2006). Liquid chromatographic analysis was performed on a C18 column (Gemini, 5µ, 150x2 mm). The injection volume was 10µl (each sample was injected twice), flow rate was 0.2 ml/min and the running time was 30 min. Prostaglandins and hydroxyl fatty acids were analysed separately. Prostaglandins analysis was performed using an acetonitrile-based gradient system of two solvents, A and B. Solvent A was: acetonitrile /water/glacial acetic acid, 45: 55: 0.02 (v/v/v); solvent B was: acetonitrile /water/glacial acetic acid, 90: 10: 0.02 (v/v/v). Hydroxy fatty acids analysis was performed using an isocratic system of two solvents, C and D. Solvent C was: methanol: water: glacial acetic acid, 80: 20: 0.02 (v/v/v); solvent D was: acetonitrile: water: glacial acetic acid, 45: 55: 0.02 (v/v/v). In order to calculate the concentration of each analyte the peak area ratio to relevant internal standard ($PGB_{2\text{-}d4}$ - 12 $HETE_{\text{-}d8}$) was calculated and read off the corresponding calibration line. The calibration lines were constructed using commercially available standards. Tables 2.1 and 2.2 show the MRM transitions and retention time for commercially available standards that are used to detect eicosanoids and hydroxy fatty acid. This analysis was performed according to the protocols published by Masoodi et al. (2006), and Nicolaou et al (2009).

Table 2.2. MRM transitions and retention times for the LC-MS/MS assay of eicosanoids

compound	MRM transitions	Retention time
PGE_1	353>317	4.47
PGD_1	353>317	4.79
$PGF_{1\alpha}$	355>311	3.69
PGB_2	333>175	9.96
PGE_2	351>271	4.47
PGD_2	351>271	5.18
$PGF_{2\alpha}$	353>193	3.77
PGJ_2	333>271	9.17
Δ^{12}-PGJ_2	333>271	9.80
15-deoxy-$\Delta^{12,14}$ PGJ_2	315>271	18.10
PGE_3	349>269	3.85
PGD_3	349>269	4.08
$PGF_{3\alpha}$	351>193	3.30
TXB_2	369>169	3.46
13,14-dihydro-15-keto PGE_1	353>335	7.42
13,14-dihydro-15-keto $PGF_{1\alpha}$	355>193	6.67
15-keto PGE_2	349>113	6.68
13,14-dihydro-15-keto PGE_2	351>333	6.89
13,14-dihydro $PGF_{2\alpha}$	355>311	4.79
13,14-dihydro-15-keto $PGF_{2\alpha}$	353>113	6.12

Table 2.3. MRM transitions and retention times for the LC-MS/MS assay of hydroxy fatty acids

compound	MRM transitions(m/z)	Retention time(min)
9-HODE	295>171	13.98
13-HODE	295>195	13.75
5-HEPE	317>115	13.18
18-HEPE	317>133	11.22
9-HEPE	317>149	12.03
8-HEPE	317>155	12.14
11-HEPE	317>167	12.03
15-HEPE	317>175	11.8
12-HEPE	317>179	12.37
5-OXOETE	317>203	18.35
14(15)EET	319>113	17.2
5-HETE	319>115	17.66
9-HETE	319>123	16.74
8-HETE	319>155	15.94
11-HETE	319>167	15.36
15-HETE	319>175	14.44
12-HETE	319>179	15.82
15-HETrE	321>221	17.55
14,15-DHET	337>207	9.97
10-HDHA	343>153	15.82
14-HDHA	343>161	15.82
13-HDHA	343>193	15.36
17-HDHA	343>201	15.02
20-HDHA	343>241	14.21

2. 5. Statistical analysis

Statistical analysis was performed with SPSS 16.0. The normality of the data was determined using the Kolmogorov-Smirnov test. One-way-ANOVA analyses were performed to compare differences in HaCaT keratinocytes and 46BR.1N fibroblasts between control groups and treated groups. The Kruskal-Wallis test was performed to compare differences in RBC and dermal PUFA content between n-3 PUFA and control groups postsupplementation. Spearman's test was used to determine correlations between RBC and dermal PUFA content. A p value of < 0.05 was considered statistically significant.

Chapter 3. Effect of n-3 PUFA on the fatty acid profile of red blood cells and dermal tissue

3.1. Introduction

Skin has a number of internal mechanisms to defend and protect it from UVR damage, including melanin production, anti-oxidant defences and enzymatic repair pathways (Sies and Stahl, 2004). Simple methods of protecting against skin damage include avoiding the midday sun, seeking shade and wearing protective clothing. However increased protection might affect vitamin D synthesis in the skin and cause vitamin D deficiency (Holick, 1996, Moloney et al., 2002). Application of sunscreens is another way to protect the skin from harmful UVR effects. These sun blocks work by scattering, reflecting or absorbing radiation but their effect is local and temporary, and the majority of UVR exposure occurs when many individuals forego sunscreen (Bech-Thomsen and Wulf, 1992) .

Dietary intervention is a safe method of protecting the skin. Omega-3 PUFAs have a beneficial role in skin health and disease prevention (Sies and Stahl, 2004). They are anti-inflammatory, and have been shown to help prevent various skin diseases such as atopic dermatitis (AD), a skin disorder common in early childhood (Kay et al., 1994). Hoppu et al (2005) found that the risk of AD was associated with high saturated fatty acid and low n-3 PUFA concentration in breast milk (Hoppu et al., 2005). A double-blind study confirmed the beneficial role of n-3 PUFA in AD. The severity of AD was found to be lower in the active group, whose members received 10 g of fish oil daily (1.8 g was EPA), compared to control subjects, who received olive oil (Bjorneboe et al., 1987). Psoriasis is an inflammatory skin disorder that can be improved by n-3 PUFA supplementation, and studies have reported a decrease in psoriasis severity

and clinical improvement following n-3PUFA supplementation (Soyland et al., 1993, Mayser et al., 2002).

The photoprotective effect of n-3 PUFA in skin disease has been supported by many studies. Rhodes et al. (2003) found, in a double-blind randomised study, that the minimal erythema dose (MED) was increased by 36% after 4g of EPA ethyl ester daily supplement was administered for three months to healthy volunteers compared to a control group supplemented with 4g oleic acid (Rhodes et al., 2003). In a second clinical trial, 20 healthy volunteers were divided into placebo and fish oil (1.2g EPA and 2.8g DHA) groups; after four weeks the results showed that, the MED was significantly increased in the fish oil supplement group compared to the placebo group (Orengo et al., 1992). A third randomised controlled trial was performed with 10 subjects: they were supplemented with 10g of fish oil daily (containing 18% EPA and 12% DHA) for three or six months and it was found that the fish oil supplement also increased the MED (Rhodes et al., 1994).

The topical effect of n-3 PUFA was studied in ten healthy human volunteers aged 25-35 years. Six sites on the surface of the forearm received 200g of fish oil extract and were exposed to 2 MED. The sites treated with fish oil extract were found to have reduced UVB-induced erythema compared to the control sites (Puglia et al., 2005). The relation between diet intake and squamous cell carcinoma (SCC) was studied, and it was found that a higher intake of n-3 PUFA reduced the risk of SCC; Moreover, the risk was decreased by increasing the ratio of n-3 PUFA to n-6 PUFA in the diet (Hakim et al., 2000). Finally, it has been reported that consumption of fish (rich in EPA and DHA) and

n-3 PUFA is related to decreased incidence of malignant melanoma (MM) (Bain et al., 1993, Veierod et al., 1997).

Human cells cannot produce the essential fatty acids LA and ALA. It has been reported that LA is the most abundant fatty acid in the human epidermis followed by arachidonic acid (Chapkin et al., 1986). Essential fatty acids are metabolised by the action of desaturation and elongation enzymes. The pathway of desaturation and elongation may function differently in various cell types. Arachidonic acid is normally found in small amounts in the human epidermis, considered to be about 3% of total epidermal fatty acids (Ziboh and Chapkin, 1988). DGLA is directly generated from dietary arachidonic acid. GLA is converted to DGLA in the human epidermis by the action of elongase enzyme (Huang and Ziboh, 2001). EPA and DHA are the two major n-3 PUFAs present in the skin, where they comprise less than 1% of total epidermal fatty acids (Ziboh and Chapkin, 1988).

The dermal skin layer is involved in homeostasis in the skin. It provides good support to the epidermis. One of the functions of essential fatty acids in the dermal layer is the production of mediators of inflammation (Ziboh and Chapkin, 1988). It has been reported that the major AA metabolite in the dermis is the anti-inflammatory 15-HETE, which can decrease the production of pro-inflammatory 12-HETE, also found in the epidermis, by inhibition of 12 lipoxygenase (Kragballe et al., 1986).

Randomised controlled trials (RCT) are the best choice for clinical studies. An RCT is a good way of assessing the effect and interference of factors such as drugs in populations or groups, and of determining whether there is a relationship between treatment and outcome. RCTs have important features

such as random selection of subjects and allocation to groups (placebo and active). The subjects participating in the study do not know which treatment they are receiving until the end of the study, and the analysis focuses on the difference in outcomes between the two groups (Sibbald and Roland, 1998).

All human studies that aim to assess the effect of dietary supplementation on peripheral tissues need circulating biomarkers. Red blood cell fatty acid content is a commonly used biomarker and is considered an index of tissue fatty acid status because the fatty acids they contain are associated with structural membrane lipids (Farquhar, 1962, Poppitt et al., 2005). Normally, RBCs contain very low amounts of EPA and DHA and their fatty acid profile is affected by diet (Arterburn et al., 2006). Moreover RBC lipids are less sensitive to dietary fluctuations than plasma lipids (Acar et al., 2012, Dougherty et al., 1987). This is because serum fatty acids reflect the composition of the last few meals, while RBCs reflect the intake for weeks or months (Glatz et al., 1989).

The fatty acid composition of RBCs has been used as a marker for many diseases. The relation of MUFA and ratio of membrane stearic to oleic acid on RBC membrane to breast cancer was studied by Pala et al. (2001), and they found that the risk of breast cancer was increased by low MUFA. A clinical study has shown that the concentration of DPA and DHA in red blood cells was low in an ischemic brain infarction patient group compared to a control group (Ricci et al., 1987). The relation between n-3 PUFA in RBCs and depression was studied by Edwards et al. (1998), who found that the severity of depression correlated negatively with n-3 PUFA in RBCs (Edwards et al., 1998). Harris's group reported that, the risk of skin squamous cell carcinoma is associated with an increase in RBC arachidonic acid (Harris et al., 2005). The risk was found to

decrease with an increase in n-3PUFA and increased ratios of n-3 PUFA to n- 6 PUFA (Hakim et al., 2000).

The clinical work described in this section was part of the study performed by our collaborators at the University of Manchester under the supervision of Prof L.E Rhodes. The study was based on the hypothesis that, diets rich in n-3 PUFA may be important to protect and keep the skin in good health. Also, epidemiological evidence indicates that n-3 PUFA could present such an opportunity, as high dietary levels are associated with reduced skin cancer risk. The study aimed to investigate the following: a) The protective role of n-3 PUFA against UVR on skin; b) the value of RBC measurements as indicator of skin content of EPA (Wallingford et al., 2012); c) the effect of oral n-3 PUFA supplementation on photoimmunosuppression in human skin (Pilkington et al., 2013). The study aims were investigated in a double-blind randomised controlled trial. Section 2.1.2.1 describes the participation in this study and 2.1.2.2 describes the study design and supplements.

The specific purposes of the present study were as follow: a) to assess the compliance of the subjects taking part in the study using RBC fatty acids as a marker; b) to analyse the fatty acid content of the dermis in the same subjects; c) to investigate the relationship between RBCs and dermal fatty acids and explore any correlation.

3.2. Methods

All relevant information is shown in section 2.3. RBC and skin sample preparation was described in section 2.3.3.1a and b. Lipid extraction in section 2.3.3.2 and section 2.3.3.5 show the preparation of fatty acid methyl esters. Statistical analysis was performed in SPSS 16.0. The normality of the data was

determined using the Kolmogorov-Smirnov test. Spearman correlation was used to assess the linear relationship between RBC and dermis.

3.3 Results

3.3.1. Analysis of the supplements (capsules)

The content of capsules containing the active (~72% EPA, 10% DHA) and placebo (glyceryl tricoprylate coprate, GTCC) supplementation were analysed by GC-FID.

N-3 supplementation was presented as EPA ethyl ester (EPAEE) and DHA ethyl ester (DHAEE). So, Active and Placebo capsules was analysed as FAEE. Figure 3.1 shows total fatty acid ethyl ester (FAEE) profile in the active and placebo capsules. It is clear that the EPA and DHA were in the active capsules and the EPA: DHA ratio was 6.3:1. Also, the results confirmed that there was no n-3 PUFA in the placebo supplement.

Placebo supplementation was presented as glyceryl tricoprylate coprate. So, Placebo and Active capsules were also analysed as FAME. This was done in order to identify the present of any other fatty acids in Active that may not have been detected as ethyl ester. The results shown in Figure 3.2 confirm that EPA and DHA were in the active capsules and EPA: DHA ratio also was 6.3:1. FAEE and FAME results confirm that EPA and DHA were present in the active capsules only and that the ratio of EPA to DHA was 6.3:1, nearly to what the manufactures specified to be 7:1. But there were some other fatty acids found by FAME analysis. These were C16:0, C18:0, C20:1n9 and C20:3n-3. The composition of EPA and DHA in the supplement was ~80% and the fatty acids C16:0, C18:0, C20:1n9 and C20:3n-3) made up the rest.

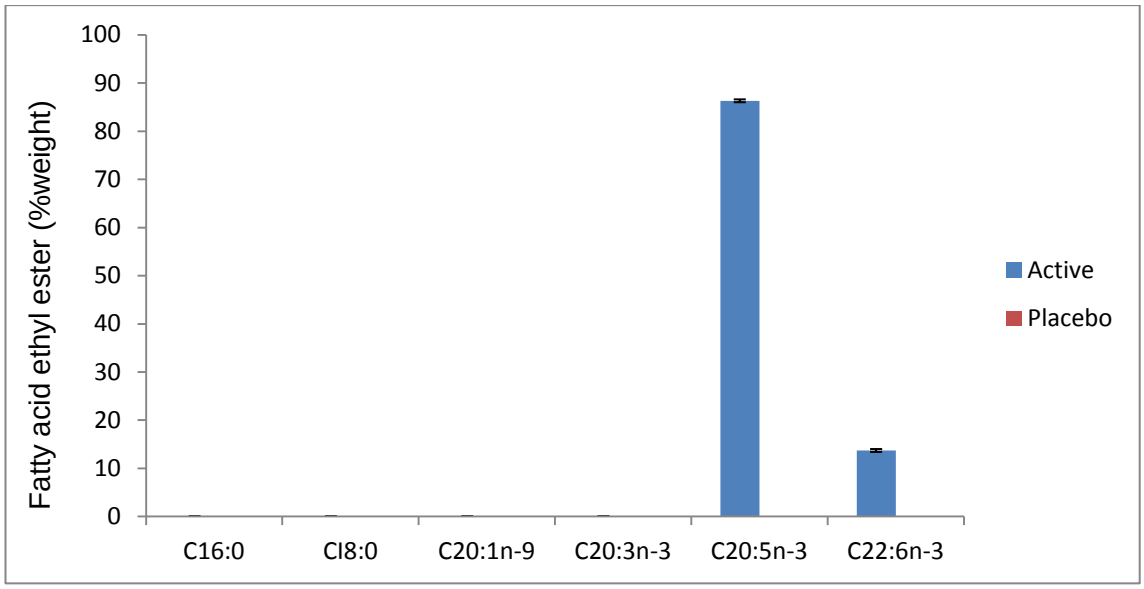


Figure 3.1. Fatty acid ethyl aster profile in ACTIVE (n-3 PUFA supplement) and placebo (glyceryl tricoprylate coprate) capsules. Results are expressed as % weight of total fatty acids (mean ± standard deviation) of 3 capsules per group, each analysed in duplicate.

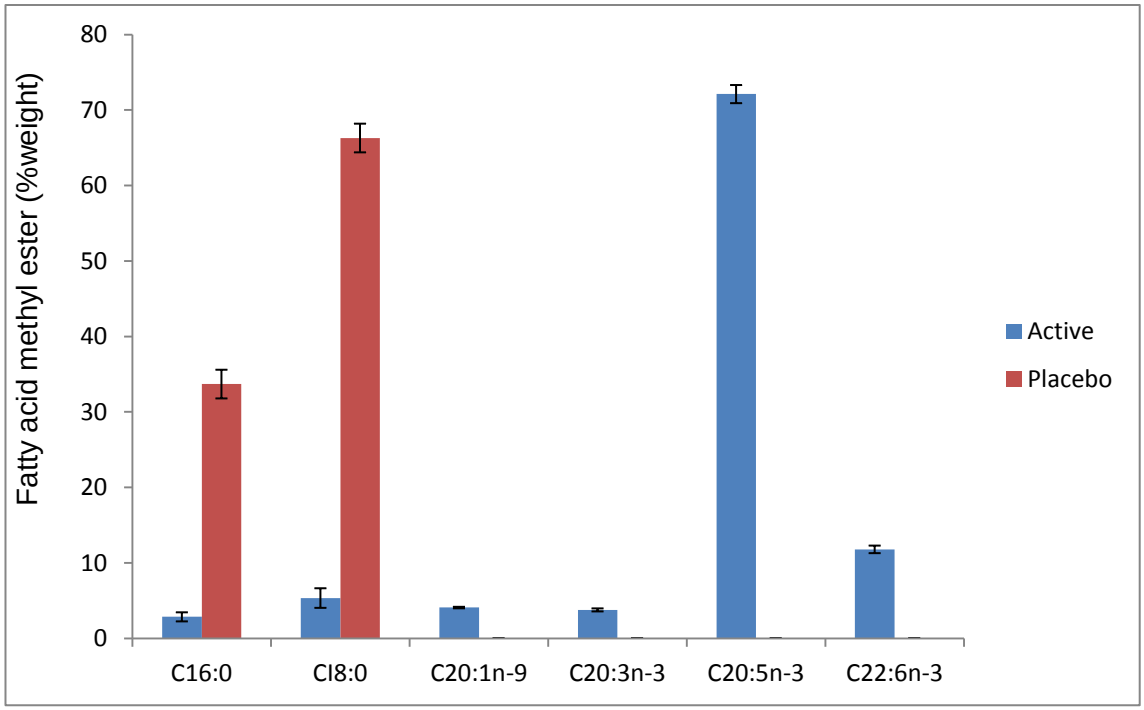


Figure 3.2. Fatty acid methyl aster profile in ACTIVE (n-3 PUFA supplement) and placebo (glyceryl tricoprylate coprate) capsules. Results are expressed as % weight of total fatty acids (mean ± standard deviation) of 3 capsules per group, each analysed in duplicate.

3.3.2. Analysis of fatty acid content of red blood cells

There were four groups in this study: pre-supplementation placebo, post-supplementation placebo, pre-supplementation active and Post supplementation active. This data is shown in Appendix 2.1. Statistically, there were no differences between pre-supplementation placebo and pre-supplementation active, therefore there two groups were combined to get the baseline reading for this study. The three groups used for data analysis were Baseline, Placebo and Active as shown in Table 3.1.

3.3.2.1 Fatty acid profile in red blood cells at baseline

The fatty acid composition of RBC is shown in Table (3.1). In detail: The saturated fatty acid group (SFA) comprised 44.27 ± 7.43% of all fatty acids. There were 10 SFA detected: C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C23:0 and C24:0, with the main ones being C16:0 at 25.53±4.39% and C18:0 at 14.89 ± 3.08%. Monounsaturated fatty acids (MUFA) were about 20.94 ± 3.29% of total RBC fatty acids. 10 MUFA were detected in this group: C14:1 C15:1, C16:1, C17:1, C18:1n-9t, C18:1n-9c, C18:1n-7, C20:1n-9, C22:1n-9 and C24:1. The main fatty acid was C18:1n.9c at 16.63 ± 2.98%. About 30% of RBC fatty acids were n-6 PUFA. This group comprised five n-6 PUFA: C18:2n.6t, C18:2n.6c, C18:3n-6, C20:3n-6 and C20:4n-6. The main PUFA was C18.2n.6c at 14.62 ± 3.28% followed by C20:4n-6 at 12.85 ± 0.53%. Five n-3 PUFA were also found and they comprised 6.82 ± 1.55% of total RBC fatty acid. These were C18:3n-3, C20:3n-3, C20:5n-3, C22:5n-3 and C22:6n-3. C22:6n-3 was found in high amount 3.57 ± 0.69% followed by C22:5n-3 and C20:5n-3 which were 1.91 ± 0.45%, 0.91 ± 0.42%, respectively.

Table 3.1 Fatty acid profile of RBC at baseline, (n=69) and post supplementation with Placebo (n=33) and n-3 supplement (Active; n=35). Results are expressed as % weight of total fatty acid (mean ± SD). a,b =p≤0.05, aa, bb =p≤0.01, aaa, bbb =p≤0.001 . (a) comparing baseline to active group and (b) comparing placebo to active group.

FATTY ACID	Baseline(n=69)	Placebo (n=33)	Active (n=35)
C12:0	0.09 ± 0.12	0.10 ± 0.02	0.10 ±0.02
C14:0	0.66 ± 0.23	0.63 ±0.11	0.63 ± 0.11
C15:0	0.16 ± 0.05	0.15 ±0.03	0.15 ± 0.03
C16:0	25.53 ± 4.39	25.18 ± 4.38	24.44 ± 4.13
C17:0	0.31 ± 0.14	0.28 ± 0.05	0.31± 0.05
C18:0	14.89 ± 4.39	14.81± 2.58	14.41± 2.44
C20:0	0.27 ± 0.12	0.25 ± 0.04	0.23± 0.04
C22:0	0.59 ± 0.32	0.48 ± 0.08	0.54 ± 0.09
C23:0	0.09 ± 0.07	0.07 ± 0.01	0.08 ± 0.01
C24:0	1.69 ± 0.88	1.46 ± 0.25	1.36 ± 0.23
ΣSFA	**44.27 ± 7.43**	**43.41 ± 7.56**	**42.26 ± 7.14**
C14:1	0.06 ± 0.03	0.01 ± 0.01	0.01 ± 0.01
C15:1	0.01 ± 0.01	0.01 ±0.01	0.01 ± 0.01
C16:1	0.84 ± 0.63	1.05 ±0.18	0.71 ±0.12
C17:1	0.01 ±0.02	0.01 ± 0.01	0.03 ±0.01
C18:1n-9t	0.13 ±0.26	0.09 ± 0.02	0.06 ±0.01
C18:1n-9c	16.63 ±2.98	16.57 ±2.88	16.67 ± 2.82
C18:1n-7	1.15 ±0.33	1.13 ±0.20	1.44 ± 0.24
C20:1n-9	0.46 ±0.93	0.45 ±0.08	0.31 ± 0.05
C22:1n-9	0.09 ± 0.05	0.09 ±.0.02	0.11±0.02
C24:1	1.61 ±0.91	1.40 ±.0.24	1.49 ± 0.25
ΣMUFA	**20.94 ± 3.29**	**20.79 ± 3.62**	**20.84 ± 3.52**
C18:2n-6t	0.06 ± 0.01	0.06±0.01	0.03 ± 0.01
C18:2n-6c	14.62 ± 3.28	15.51± 2.70	13.73 ± 2.32[a,bb]
C18:3n-6	0.19 ± 0.18	0.16 ±.0.03	0.11 ± 0.02[**aaa,bb**]
C20:3n-6	1.88 ± 0.53	1.97 ± 0.34	1.56 ± 0.26[**aaa,bbb**]
C20:4n-6	12.85 ± 0.53	12.77 ± 2.22	11.84 ± 2.00[a,b]
ΣN-6PUFA	**29.61 ± 5.53**	**30.46 ± 5.30**	**27.28 ± 4.61**[**aaa,bbb**]
C18:3n-3	0.40 ±.0.32	0.37 ±.0.07	0.31 ± 0.05
C20:3n-3	0.02 ± 0.05	0.03±.0.01	0.02 ± 0.001
C20:5n-3	0.91 ± 0.42	0.92 ±0.16	3.61 ± 0.61[**aaa,bbb**]
C22:5n-3	1.91 ± 0.45	1.93 ±.0.34	3.19 ± 0.54[**aaa,bbb**]
C22:6n-3	3.57 ±.0.69	3.53 ±0.61	4.20 ± 0.71[**aa,bb**]
ΣN-3PUFA	**6.82 ± 1.55**	**6.79 ± 1.18**	**11.34 ±1.92** [**aaa,bbb**]
C20:2	0.19 ± 0.23	0.26 ± 0.05	0.20 ±0.03
C22:2	0.07 ±0.01	0.04 ± 0.01	0.06 ±0.01

3.3.2.2. Effect of placebo on red blood cell fatty acid profile

There was no statistically significant difference in the levels of fatty acids in the Placebo group compared to the Baseline group (Table 3.1).

3.3.2.3. Effect of n-3PUFA supplementation on red blood cell fatty acids

Statistical analysis has shown that there was not any effect of the n-3 PUFA supplement on the saturated and monounsaturated fatty acids in red blood cells, but there was an effect on the n-6 and n-3 PUFA. Specifically:

A decrease in n-6 PUFA was observed in the Active group compared to the Baseline and Placebo groups. A significant decreased from 29.6% ± 5.5 at baseline to 27.3 ± 3.0% in active group ($p \leq 0.001$). Also a significant decreased from 30.5% ± 5.0 at placebo to 27.3 ± 3.0% in active group ($p \leq 0.001$). These changes are due to changes in the level of individual fatty acids. Specifically: The concentration of C18:2 n-6c and C18:3 n-6 were significantly decreased in the Active group compared to Baseline ($p \leq 0.05$, and $p \leq 0.001$, respectively). Also they were significantly decreased in the Active group compared to Placebo ($p \leq 0.006$, and $p \leq 0.005$, respectively) (Table 3.1). The amount of C20:3n-6 was significantly decreased in the Active group compared to Baseline ($p \leq 0.001$) and Placebo groups ($p \leq 0.001$). Also a significantly decreased in the concentration of C20:4 n-6 in the Active group compared to Baseline ($p \leq 0.05$) and Placebo groups ($p \leq 0.05$). Results shown in Table 3.1.

Finally the results show that the amount of n-3 PUFA increased in the Active group. It was significantly increase from 6.80 ± 1.6% at the Baseline and Placebo group to 11.34 ± 1.92% in Active group ($p \leq 0.001$). Table 3.1 shows the effect of n-3 PUFA supplementation on C20:5n-3. There was a significant increase in the Active group when compared to Baseline ($p \leq 0.001$) and Placebo

groups (p≤0.001). Also, a significant increase was observed in the amount of C22:5n-3 after n-3 PUFA supplementation (Active) compared to Baseline (p≤0.001) and Placebo group (P≤0.001). Finally, An increase of C22:6n-3 concentration was observed in the Active group after n-3 PUFA supplementation. This was significantly increased compared to Baseline and Placebo groups (p≤0.001 and p≤0.01, respectively).

3.3.3. Analysis of fatty acid content of dermis

There were four groups in this study: pre-supplementation placebo, post-supplementation placebo, pre-supplementation active and post-supplementation active. This data is shown in Appendix 2.2. Statistically, there were no differences between pre-supplementation placebo and pre-supplementation active; therefore the two groups were combined to get the baseline reading for this study. The three groups used for data analysis were baseline, placebo and active as shown in Table 3.2.

3.3.3.1. Fatty acid profile of dermis

The fatty acid composition of human dermis is shown in Table 3.2. In detail: Saturated fatty acids were 27.06 ± 9.54% of total fatty acids and it was less than it was in RBC (Figure 3.3). Ten SFA were detected : C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C23:0 and C24:0, with the main fatty acid being C16:0.

Table 3.2 Fatty acid profile of human dermis at baseline, (n=33), placebo (n=14) and post n-3 supplement (active)(n=19). Results are expressed as % weight of total fatty acid (mean ± SD). a,b = p≤0.05 aaa,bbb = p≤0.001 . (a) comparing baseline to active group and (b) comparing placebo to active group.

Fatty acid	Baseline(n=33)	Placebo(n=14)	Active(n=19)
C12:0	0.18 ± 0.37	0.21 ± 0.05	0.17 ± 0.04
C14:0	1.83 ± 0.57	1.82 ± 0.49	1.76 ± 0.40
C15:0	0.23 ± 0.09	0.24 ± 0.06	0.21 ± 0.05
C16:0	21.05 ± 7.65	20.52 ± 5.48	19.06 ± 4.37
C17:0	0.20 ± 0.17	0.23 ± 0.06	0.13 ± 0.03
C18:0	3.27 ± 2.4	3.72 ± 0.99	2.57 ± 0.59
C20:0	0.18 ± 0.14	0.13 ± 0.03	0.16 ± 0.04
C22:0	0.03 ± 0.01	0.04 ± 0.01	0.01 ±0.01
C23:0	0.02 ± 0.01	0.01 ± 0.01	0.00 ±0.00
C24:0	0.06 ± 0.06	0.07 ± 0.02	0.05 ± 0.01
ΣSFA	**27.06 ± 6.78**	**26.97 ± 7.71**	**24.13 ± 5.54**
C14:1	0.52 ±0.35	0.49 ± 0.13	0.57 ± 0.13
C15:1	0.03 ± 0.05	0.02 ± 0.01	0.01 ± 0.01
C16:1	10.0 ± 4.23	8.21 ± 2.19	10.38 ± 2.38
C17:1	0.18 ± 0.05	0.17 ± 0.04	0.21 ± 0.05
C18:1n-9t	0.85 ±0.25	0.72 ± 0.19	0.34 ± 0.08
C18:1n-9c	43.78 ± 9.42	46.01 ±12.30	46.82 ± 10.74
C18:1n-7	3.08± 1.50	3.28 ± 0.88	2.75 ± 0.63
C20:1n-9	0.40 ± 0.12	0.44 ±0.12	0.39 ± 0.09
C22:1n-9	0.05 ± 0.01	0.06 ± 0.02	0.03 ± 0.01
C24:1	0.03 ± 0.01	0.04 ± 0.01	0.02 ± 0.01
ΣMUFA	**58.90 ± 9.80**	**59.43 ± 15.88**	**61.52 ± 14.11**
C18:2n-6t	0.14 ± 0.11	0.14 ± 0.04	0.10 ± 0.02
C18:2n-6c	10.96 ± 2.81	10.99 ± 2.94	11.63 ± 2.67
C18:3n-6	0.06 ± 0.02	0.08 ± 0.02	0.07 ± 0.02
C20:3n-6	0.21 ± 0.09	0.22 ± 0.06	0.19 ± 0.04
C20:4n-6	0.66 ± 0.32	0.66 ± 0.18	0.61 ± 0.14
ΣN-6PUFA	**12.47 ± 3.33**	**12.08 ± 3.23**	**12.60 ± 2.89**
C18:3n-3	0.75 ± 0.26	0.69 ± 0.19	0.81 ± 0.19
C20:3n-3	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
C20:5n-3	0.06 ± 0.32	0.06 ± 0.02	0.12 ± 0.03 ^{aaa,bbb}
C22:5n-3	0.20 ± 0.74	0.19 ±0.05	0.24 ± 0.05^{a,b}
C22:6n-3	0.18 ± 0.09	0.16 ± 0.04	0.21 ± 0.05
ΣN-3PUFA	**1.32± 0.37**	**1.14 ±0.31**	**1.42 ±0.33**
C20:2	0.37 ± 0.08	0.36 ± 0.10	0.32 ± 0.07
C22:2	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01

The monounsaturated fatty acids group was found at higher levels (58.90±9.80%) and it is higher than in RBC (Figure 3.3). Ten MUFA were detected in this group; C14:1 C15:1, C16:1, C17:1, C18:1n-9t, C18:1n-9c, C18:1n-7, C20:1n-9, C22:1n-9 and C24:1. C18:1n.9c was the main fatty acid.

N-6 PUFA comprised about 12.47±3.33% of total dermal fatty acids with 5 PUFA present: C18:2n.6t, C18:2n.6c, C18:3n-6, C20:3n-6 and C20:4n-6. C18.2n.6c was the main n-6 PUFA with 10.96±2.81%. The n-6 PUFA levels were lower than in RBC (Figure 3.3)

Five n-3 PUFA, C18:3n-3, C20:3n-3, C20:5n-3, C22:5n-3, C22:6n-3 were detected in n-3 PUFA group and comprised 1.23±0.37% of total dermal fatty acid. The levels of n-3 PUFA were less than in RBC (Figure 3.3).

3.3.3.2. Effect of supplementation on dermal fatty acids

There was no statistically significant difference in the levels of fatty acids in the placebo group comparing to the baseline group.

Also, there was no effect of n-3PUFA supplementation on SFA, MUFA and n-6 PUFA. However, n-3 PUFA was affected by n-3PUFA supplementation. Specifically: C20:5n-3 and C22:5n-3 increased after 3 months of n-3PUFA supplementation. C20:5n-3 was significantly increased from 0.06 ± 0.02% at the baseline and Placebo group to 1.12 ± 0.03% at Active group, and this was statistically significant ($p=0.001$ and $p\leq0.001$, respectively) (Table 3.2). Also, C22:5n-3 was significantly increase in active group compared to baseline ($p\leq0.05$) and placebo ($p\leq0.05$). (Table 3.2)

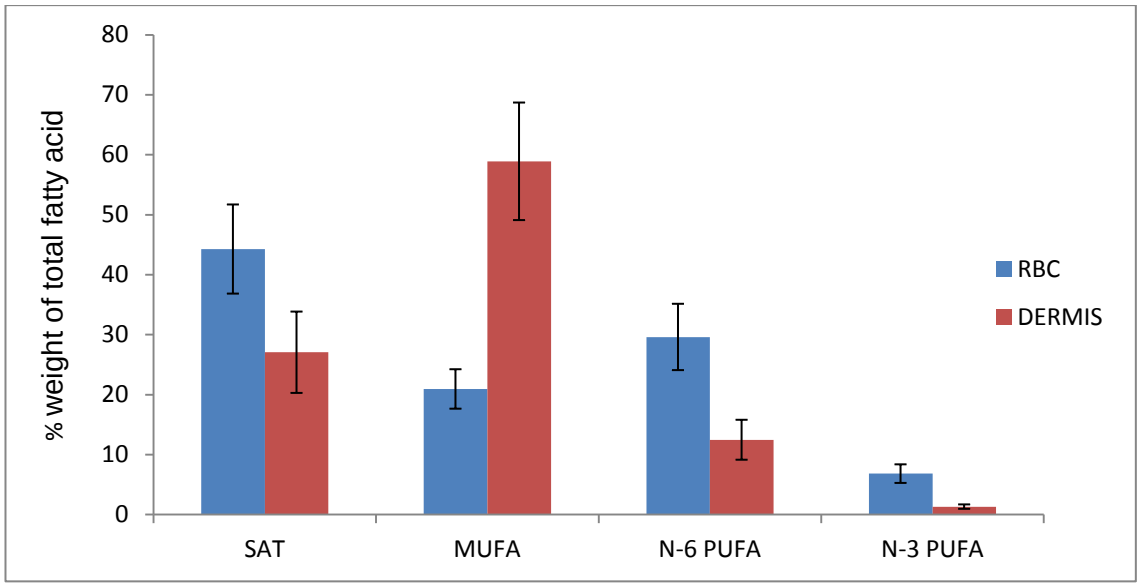


Figure 3.3. Fatty acid groups of red blood cell (RBC, n=69) and dermis (n=33) at baseline. Results are expressed as % weight of total fatty acids (mean ± SD). SFA= saturated fatty acids. MUFA= monounsaturated fatty acids, N-6 PUFA= n-6 polyunsaturated fatty acids. N-3 PUFA= n-3 polyunsaturated fatty acids.

3.3.4. Correlation of fatty acids in dermis and RBC

Correlation test (Spearman correlation test for non parametric data) was run between the main PUFA of the samples in the Placebo and Active groups before and after supplementation. Results in Figure 3.4 suggest that AA, EPA DPA and DHA showed no significant correlation between dermis and RBC before and after supplementation at Placebo group.

Among all PUFA in the Active group, AA shown a significant correlation at post (r=0.581, p≤0.011) supplementation. Although, the results show that the correlation of EPA was not statistically significant, it is clear that the EPA data were in the lower left of the scatter plot at pre-active supplementation group. In contrast the n-3PUFA supplement takes the data in the upper right of the scatter plot (Figure 3.5).

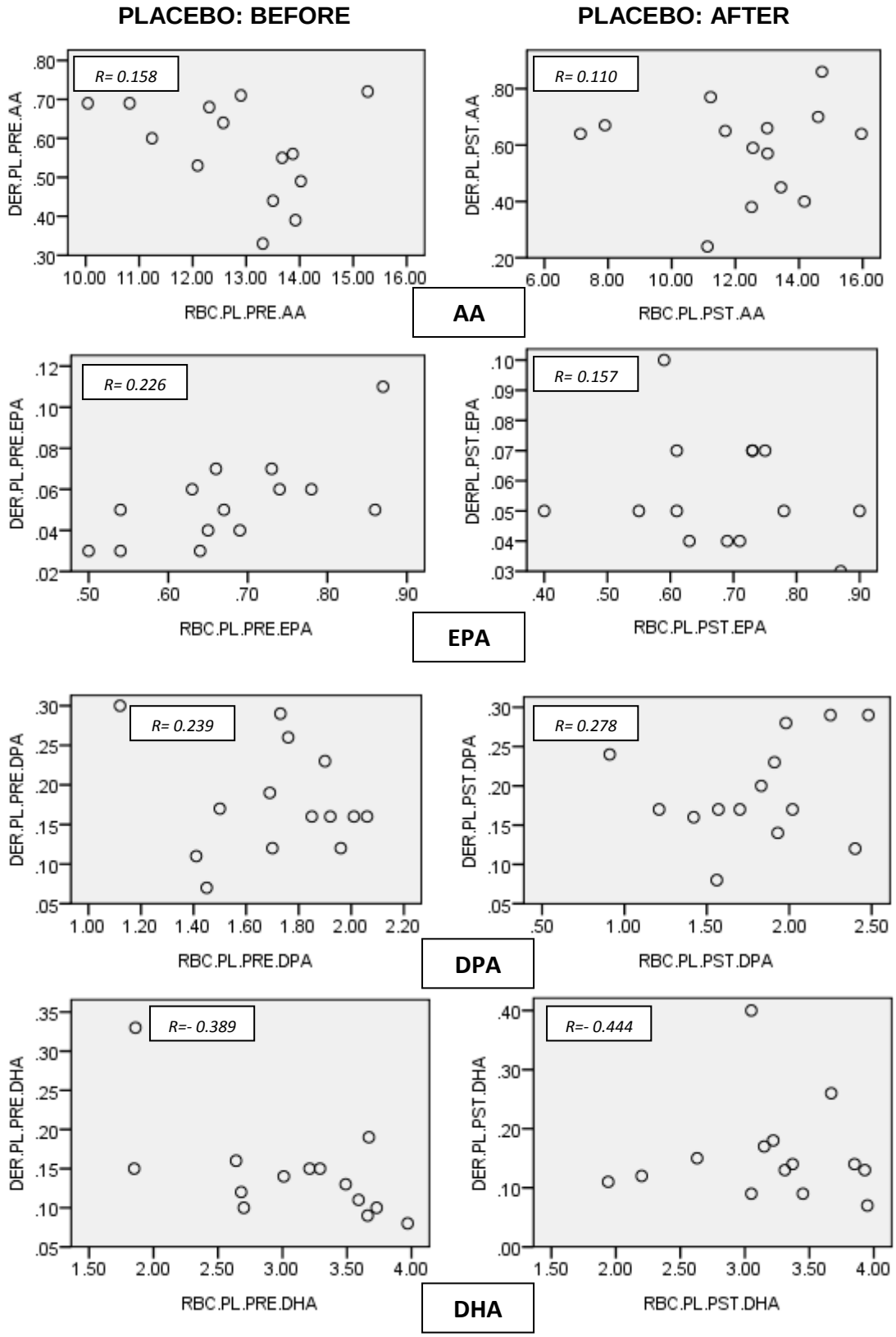


Figure 3.4. Correlation of dermal and red blood cells (RBC) level of arachidonic acid (AA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexa-enoic acid (DHA) before and after placebo supplementation (n=14 subjects). DER=dermal. RBC=, PL.PRE= Placebo pre supplement. PL.PST= Placebo post supplement.

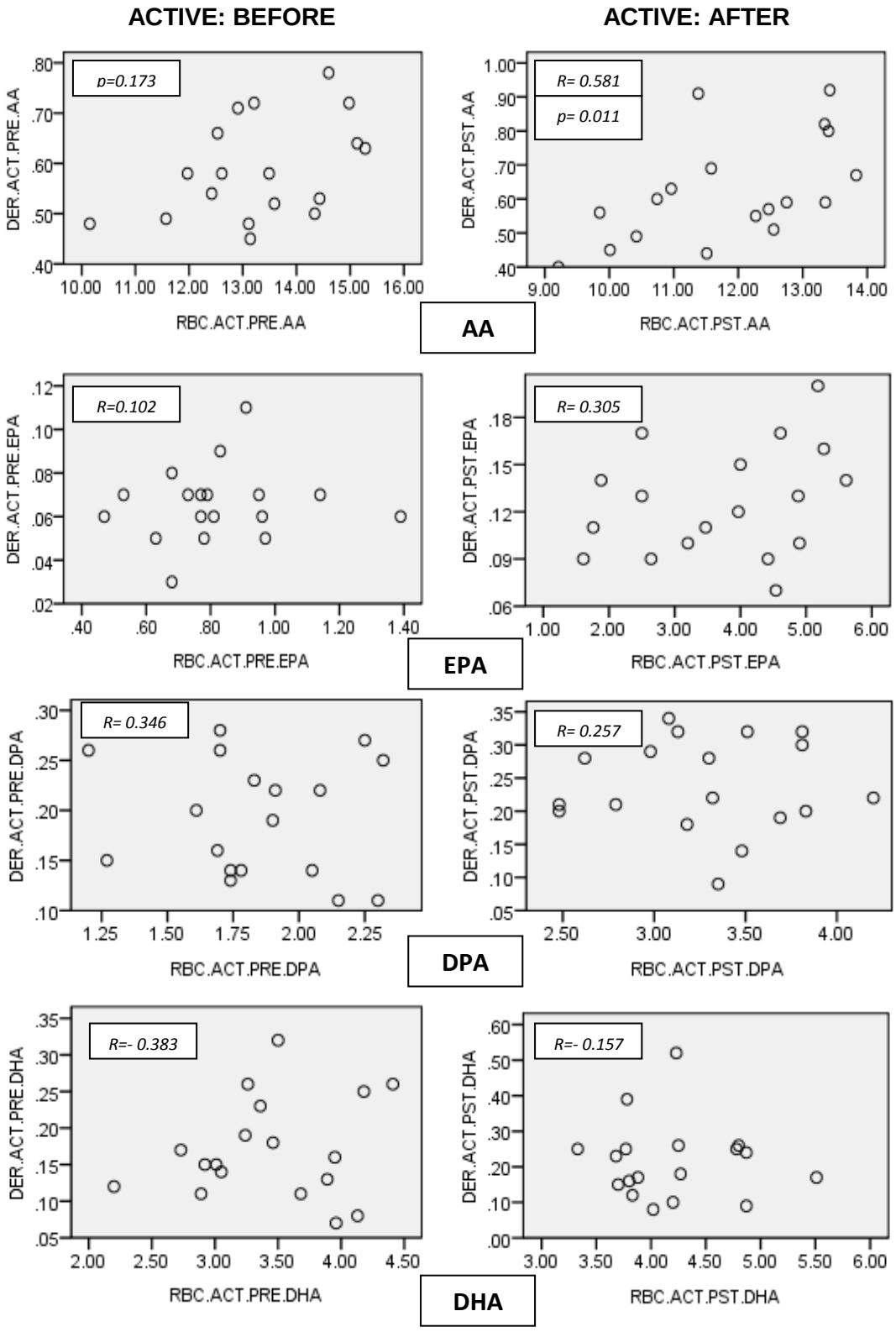


Figure 3.5. Correlation of dermal and red blood cells (RBC) level of arachidonic acid (AA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexa-enoic acid (DHA) before and after active supplementation (n=19 subjects). DER=dermal. ACTV.PRE= Active pre supplement. ACTV.PST= Active post supplement.

3.3.5. Fatty acid profile of epidermis

Five samples of epidermis were available for fatty acid analysis. The detailed fatty acid profile for epidermis, dermis and RBC is shown in Appendix 2.3.

Figure 3.6 shows that SFA were higher in RBC (42%) followed by epidermis (37%) and dermis (25%). The main SFA C16:0 was at the same level in epidermis and dermis (20%) and at RBC(24%). C18:0 was lower in dermis (3%), then in epidermis (10%) and RBC (14%).

High amounts of MUFA were found in dermis (61%) followed by epidermis (46%) and RBC (22%). C18:1n-9c was found to be the main FA in this group with a high level in dermis (45%) followed by epidermis (35%) and lower RBC (17%) (Figure 3.7).

N-6 PUFA was higher in RBC (30%) followed by epidermis (14%) and dermis with (12%). The amount of C18:2n-6c was at the same concentration at epidermis and dermis with (11%) and RBC (15%). C20:4n-6 was found at low level in dermis with (0.7%) and epidermis (2.3%) and higher in RBC (14%) (Figure 3.8).

N-3 PUFA was higher in RBC (6.8%) and lower in epidermis (2.3%) and dermis (1.2%). The amount of C20:5n-3 was also at low concentration in the dermis (0.07%), epidermis (0.61%) and RBC (0.77%). C22:5n-3 level were also low in dermis was also low (0.18%), epidermis (0.41%) and at RBC (1.78%). C22:6n-3 was found at a high level in RBC (3.83%) but low in epidermis (0.38%) and at dermis (0.19%) (Figure 3.9).

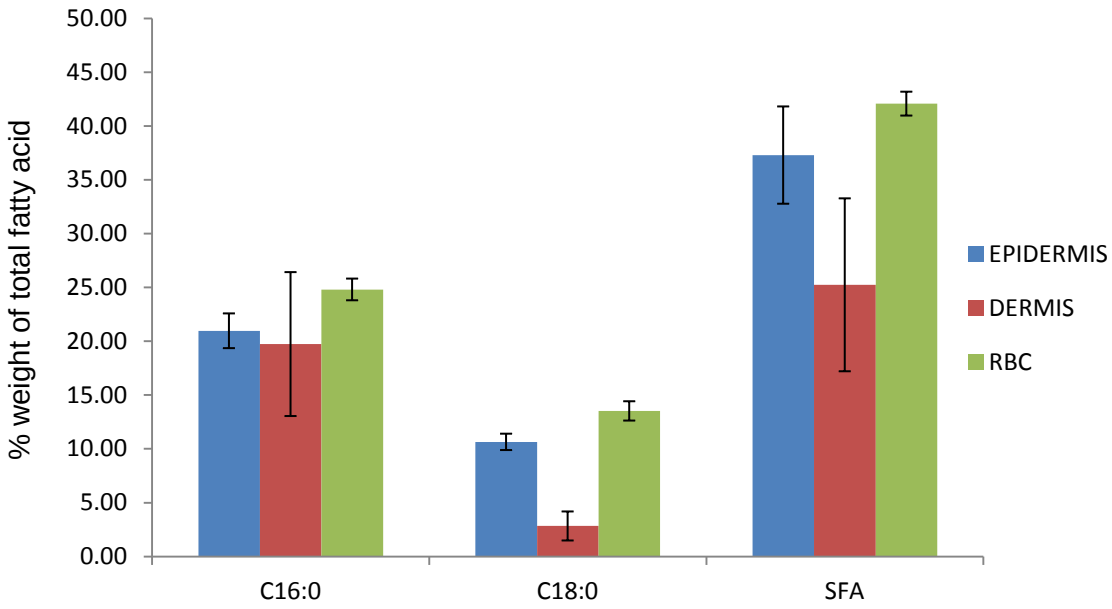


Figure 3.6.Level of saturated fatty acids (SFA), palmitic acid (C16:0) and stearic acid (C18:0) on epidermis, dermis and red blood cells (RBC). Results are expressed as % weight of total fatty acids (mean ± SD of 5 volunteers, each analysed in duplicate).

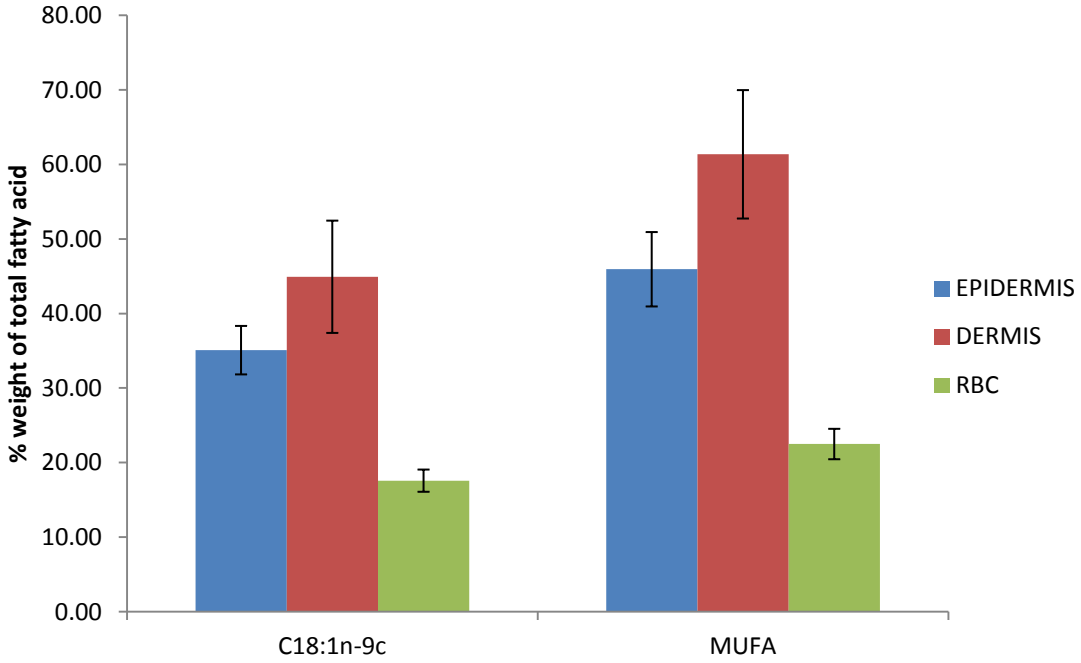


Figure 3.7.Level of monounsaturated fatty acid (MUFA), oleic acid (C18:1n-9c) on epidermis, dermis and red blood cells (RBC). Results are expressed as % weight of total fatty acids (mean ± SD of 5 volunteers each analysed in duplicate).

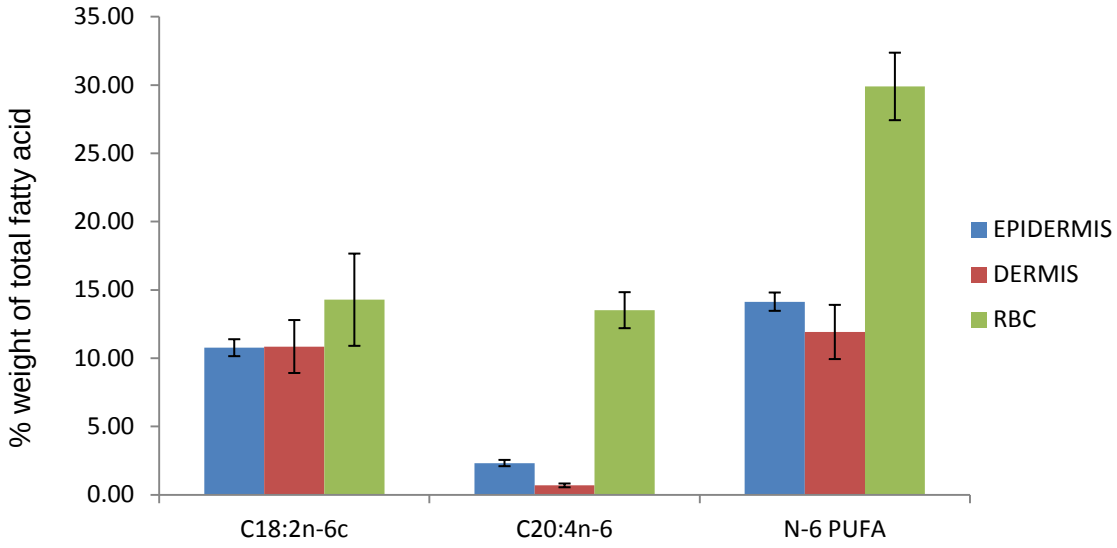


Figure 3.8.Level of n-6 polyunsaturated fatty acid (n-6 PUFA), linoleic acid (LA, C18:2n-6c) and arachidonic acid (AA, C20:4n-6) on epidermis, dermis and red blood cell (RBC). Results are expressed as % weight of total fatty acid (mean ± SD) of 5 volunteers each analysed in duplicate.

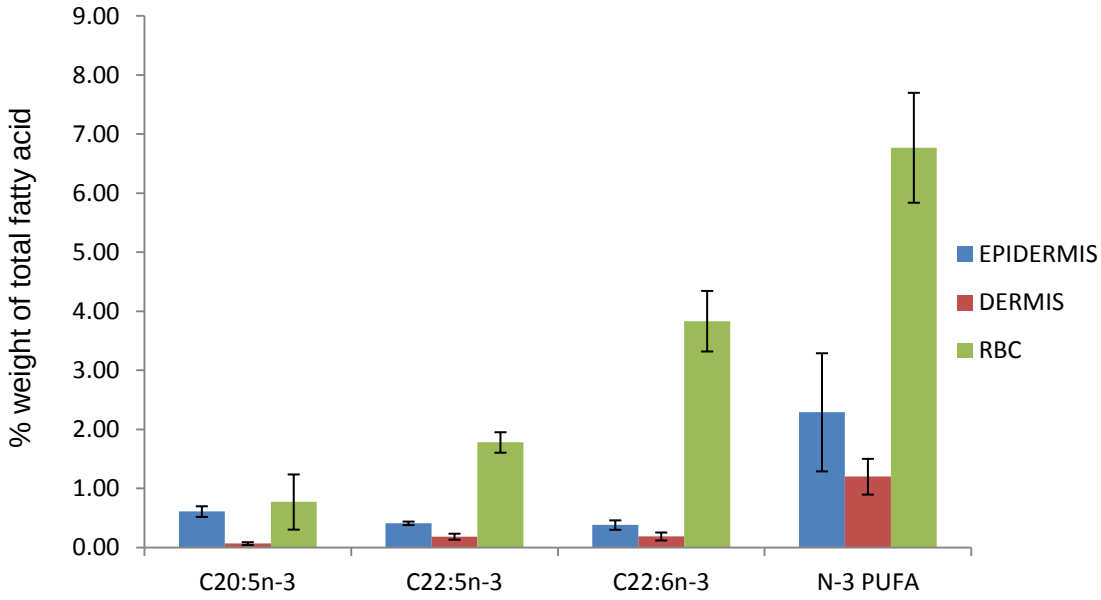


Figure 3.9. Level of n-3 polyunsaturated fatty acid (n-3 PUFA), eicosapentaenoic acid (EPA, C20:5n-3), docosapentaenic acid (DPA, C22:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) on epidermis, dermis and red blood cell (RBC). Results are expressed as % weight of total fatty acids (mean ± SD) of 5 volunteers each analysed in duplicate.

3.4. Discussion

In clinical studies testing drugs or nutritional supplementation, it is important to have some measure of compliance or relevant biomarkers. It has been confirmed by many studies that the fatty acid composition of RBCs, white blood cells and plasma can change in response to n-3PUFA supplementation (Poppitt et al., 2005, Harris et al., 2007, Morlion et al., 1996) Furthermore, it has been reported that the fatty acid profile of RBCs is less changeable, and it has been a better biomarker than white blood cells (Witte et al., 2010) and plasma (Harris and Thomas, 2010).

The baseline results in this study shown that 32 fatty acids were detected in RBCs. The SFA group was the higher concentration; palmitic acid (C16:0) and stearic acid (C18:0) were the main SFAs. Harris et al (2005) also reported that palmitic acid (27%) and stearic acid (17%) were the main SFAs in RBCs (Harris et al., 2005); these concentration were higher than in our study, were they were 25% and 14.8%, respectively (Table 3.1). A higher amount of palmitic acid (44%) and a lower amount of stearic acid (11%) than those recorded in our results were found by Ricci et al. (1987). Moreover, in the control group (women (n=18) and men (n=2)) it was found that C16:0 (20.62%) and C18:0 (15.6%) were the main SFAs (Miranda et al., 2011). Palmitic acid was lower and stearic acid was higher compared to our results (Table 3.1) It has also been reported that, among the Shanghai women, the amount of palmitic acid (18%) was lower than that found in the present study (Manchester women) while the amount of stearic acid (13%) was mostly the same (Table 3.3) (Shannon et al., 2007). In contrast, among Swedish women the amount of palmitic acid (27%) was higher

and the amount of stearic acid (11%) was lower than in the current study (Table3.5) (Wirfalt et al., 2004).

In this study, oleic acid has been found to be the main MUFA in RBCs at 17%. Certain studies reported OA as the main MUFA; higher amounts than found in this study were reported by Harris et al. (2005), Ricci et al. (1987) and Wirfalt et al. (2004), while lower amounts than found in this study were reported by Shannon et al. (2007), Gemma Miranda et al. (2011).

Table 3.3. Composition of RBC fatty acids in Manchester, Shanghai and Swedish women

Fatty acids	**Manchester (present study)**	**Shanghai (Shannon et al., 2007)**	**Sweden (Wirfalt et al., 2004)**
C16:0	25.53 ± 4.39	18.73 ± 1.04	27.8 ± 1.03
C18:0	14.89 ± 4.39	13.93 ± 0.95	11.4 ± 0.81
ΣMUFA	**20.94 ± 3.29**	**19.18 ± 2.27**	
C18:1n-9c	16.63 ± 2.89	10.38 ± 1.22	18.0 ± 1.01
ΣN-6PUFA	**29.61 ± 5.53**	**28.13 ± 2.42**	**28.13 ± 2.42**
C18:2n-6c	14.62 ± 3.28	12.17 ± 2.84	13.4 ± 1.39
C20:4n-6	12.85 ± 0.53	12.08 ± 1.19	12.4 ± 1.12
ΣN-3PUFA	**6.82 ± 1.55**	**7.69 ± 1.07**	
C22:6n-3	3.57 ± 0.69	4.92 ± 0.85	6.47 ± 1.06
C22:5n-3	1.91 ± 0.45	1.86 ± 0.34	2.62 ± 0.29
C20:5n-3	0.91 ± 0.42	0.60 ± 0.22	1.71 ± 0.58

The results presented in Table 3.1, show that LA (15%) is the main n-6 PUFA in RBCs followed by AA (13%). The amount of LA in this study was higher than reported in other studies (Wirfalt et al., 2004, Shannon et al., 2007) but the amount of AA was similar to that found in these studies (Table 3.1). Low amounts of LA (12%) and AA (3.8%) were reported by Ricci et al. (1987). In contrast to our result AA was found to be the main n-6PUFA (13.3%) followed by LA (10%) and both were lower than in our study (Gemma Miranda et al., 2011). Furthermore, the amount of AA (14.6%) was higher and that of LA (13.0%) was lower compared to our results (Harris et al., 2005).

In the current study the main n-3 PUFA in RBCs was DHA (3.57%) followed by DPA (1.91%) and EPA (0.91%). A similar result was reported by other groups (Harris et al., 2005, Wirfalt et al., 2004, Barcelo-Coblijn et al., 2008). Another group reported a lower value of DPA (1.19%; main n-3 PUFA) followed by DHA (0.39%), and a smaller amount of EPA (0.15%) than was observed in this study (Ricci et al., 1987). In a higher amount than found in our study, DHA (3.71%) was the main n-3 PUFA, while EPA (0.42%) was lower than in our result (Miranda et al., 2011).

The profile of dermal fatty acids shows that the SFA comprised about 27% of total fatty acids; this was not as high as in RBC cells and C16:0 was the main SFA in the dermis (21%). MUFA were found in higher amounts in the dermis where they comprised about 59% of total fatty acids. However this was not the same in RBCs where they comprised only 21% of total fatty acids. C18:1n-9c is the main MUFA in the dermis at 74.20% of MUFA. The concentration of n-6 PUFA in RBC was 29.6%, and was higher than the level found in the dermis

where it was only 12%. C18:2n-6c was the main fatty acid in the dermis, comprising 91.3% of the total n-6 PUFA.

The fatty acid profile of the epidermis was studied in only five samples that were available for this study. The composition of fatty acids in the epidermis is shown in Appendix 2.3. The results suggest that C18:1n-9c was the most abundant (35%) of total fatty acids in the epidermis, followed by C16:0 (21%), C18:2n-6c (10.77%) and C18:0 (10.65%).The amount of n-3 PUFA was higher in the epidermis (2.29%) than in the dermis (1.20%). In the epidermis, the concentration of EPA (0.61%) was higher than DPA (0.41%) and DHA (0.38%). In contrast, dermis DHA (0.19%) was higher than DPA (0.18%) and EPA (0.07%). Ni Raghallaigh et al (2012) reported that C16:0 (22%) is the main SFA in the skin surface lipid layer and C16:1(21%) is the main MUFA, rather than C18:1 as shown in this study. They also show that the concentration of DHA (0.30%) was higher than EPA (0.21%) (Ni Raghallaigh et al., 2012). C16:0 and C18:0 were found in high amounts in both living and stratum corneum of human sole skin epidermis (Ansari et al., 1970). A study of human epidermis observed that C16:0 (14%) was the main SFA, followed by C18:0 (11.06%), both of which were lower than in our study. OA (15.12%) was the main MUFA, in a low amount compared to our result. LA (21.52%) was the main n-6 PUFA followed by AA (6.22%) both of which were higher than found in the current study (Appendix 2.3) (Chapkin et al., 1986).

A variety of scientific associations and regulatory bodies have recommended a greater intake of n-3 PUFA (EPA and DHA) to help reduce the risk of disease in population. Determining the effect of n-3 PUFA supplement on fatty acid composition in RBCs and skin dermis was one target of this study.

The profile of the RBC fatty acids of the Active group was compared to that of the Baseline group to investigate whether there was any alteration to the fatty acid profile after three months of n-3 supplementation.

The result shows that PUFA composition of RBC changed after n-3 PUFA supplementation. Among n-6 PUFA, only C18:2n-6t was not significantly affected. C18:2 n-6c, GLA, DGLA and AA significantly decreased after three months of mainly EPA supplementation. By contrast, EPA, DPA and DHA were significant increased after 3 months of EPA and DHA supplementation. This result was in agreement with the literatures where it was reported that the n-3 PUFA content of RBCs increased linearly in relation to n-3 PUFA supplementation (Witte et al., 2010). Prisco et al reported that EPA, DPA and DHA significantly increased after four months of EPA (2.04g) and DHA (1.4g) ethyl ester (4g/d) supplementation (Prisco et al., 1996). Palozza et al found that after 30 and 180 days of n-3 supplementation, the PUFA pattern of the RBCs changed and the concentration of both EPA and DHA increased (Palozza et al., 1996). Barcelo Coblijn also reported that the supplementation of fish oil (0.6 and 1.2 g/d) led to an increase in n–3 PUFA content in RBCs compared with the placebo group (Barcelo-Coblijn et al., 2008). A significant increase in n–3 PUFA concentration in RBCs after consumption of 6g fish oil/d compared with a placebo group receiving microcrystalline cellulose was reported by (Oostenbrug et al., 1997). Cao et al (2006) have also reported that after 8 weeks of fish oil supplement (EPA 1296 mg, DHA 864 mg) at 6 capsules per day plus one capsule of vitamin E (400 IU), EPA, DHA and DPA increased significantly in RBC membranes (Cao et al., 2006).

The fatty acid profile in skin dermis did not change in a way similar to RBCs. Among all fatty acids in the dermis only EPA and DPA significantly increased after three months of n-3 PUFA supplementation (Table 3.2). Few studies have reported the effect of n-3 PUFA supplementation in skin fatty acids. Rhodes et al (1994) reported that in skin shave biopsies (predominantly epidermis with some dermal tissue) the EPA content increased after three months' supplementation with 10g of Max EPA (18% EPA, 12% DHA) daily. The total n-3 PUFA increased from 1.8% of total fatty acid in the epidermis to 24.2% after three months of n-3 PUFA supplementation (Rhodes et al., 1994). Rhodes et al. (2003) also reported an eightfold increase in skin (epidermis and dermis) EPA concentration after supplementation with 4g/day 95% EPA ethyl for three months (Rhodes et al., 2003).

The RBC and dermal PUFA data produced another finding: RBC EPA has been elongated and desaturated to DPA and DHA (Table 3.1) but this was not observed in the skin samples, where we only see an increase in EPA which had been elongated to and increased to DPA but not DHA. This may be because the amount of DHA in the supplement (1/7th of the supplement) was not sufficient to alter skin DHA content. The inability of skin to desaturate DPA to DHA is an indication of the lack of desaturase activity in the human epidermis. It has been suggested that the absence of epidermal desaturase activity may be result of the freezing of tissue specimens or contamination of the microsomal preparations (Chapkin et al., 1986). Another observation from this study is the lack of effect on arachidonic acid levels in both RBCs and skin, suggesting that the supplement did not reduce arachidonic acid availability for PGE_2 (and other eicosanoids) and may not have anti-inflammatory activity.

In summary, after three months of n-3 PUFA supplementation the volunteers who participated in this study were regularly taking the supplement. This was confirmed by an increase in the levels of EPA, DPA and DHA in RBCs and also by an increase in EPA and DPA in skin. No significant correlations between n-3 PUFA in RBCs and skin were found. Moreover, the n-3 PUFA in circulation is not a good measure of skin bioavailability of these long-chain n-3 PUFA.

Chapter 4. The effect of PUFA and UVR on skin cell viability

4.1. Introduction

The effect of UVR on humans, animals and bacteria has been widely studied. Data from experimental as well as epidemiological studies have provided strong evidence that UVR is involved in the pathogenesis of many diseases. Depending on the wavelength and dose of UVR, the effect of UVR on human health can be useful or harmful (Ohnaka, 1993). Small doses of UVR are functionally useful for humans, essentially in vitamin D synthesis (MacKie, 2000). UVR is also used in the medical field to treat many diseases such as psoriasis, eczema, rickets and jaundice (EUROSKIN, 2000).

The harmful effects of UVR depend on the wavelength of the radiation. In general, harmful effects result in photocarcinogenesis, photodamage, damage to the eyes, DNA damage, modification of the skin's immune system, and chemical hypersensitivity (Ohnaka, 1993, Nataraj et al., 1995). In skin, all main types of skin cancer, (basal cell carcinoma, squamous cell carcinoma and melanoma) are linked to UVR in sunlight (IARC, 1992). Overall, toxic effects of UVR from natural sources, artificial lamps and sun beds are major risk factors for human skin cancer.

The sun is the main source of natural UVR. The UVB component of sunlight is about 5%-10% of total UVR (280 and 320 nm) and 90%-95% of UVR is UVA (320-400nm). Although the sun is the main source of terrestrial UVR, artificial sources such as welding arcs, germicidal lamps and tanning lamps all emit UVR. A Philips TL12 UV lamp, with an emission range of 270 – 400 nm and a peak of 311nm was used in this study. The same source of UVR was used to irradiate HaCaT cells in other similar studies (Storey et al., 2007).

Yoo et al (2008) used an FS20 Lamp that emits light at a continuous spectrum from 270 to 390 nm, with peak emission at 313 nm. UVB accounts for 65% of the light emitted by this lamp (Yoo et al., 2008).

N-3 PUFAs play an important role in the growth and development of human cells. Diet supplementation with EPA and DHA is known to have various useful effects such as prevention of cardiovascular diseases (Kris-Etherton et al., 2002), and reduction in blood triglycerol levels (Weber and Raederstorff, 2000), reduction in platelet aggregation and blood pressure (Holub, 2002). Studies in humans have reported that n-3 PUFAs have anti-inflammatory properties and may protect UVB-exposed skin (Rhodes et al., 2003, Rhodes et al., 1994, Orengo et al., 1992).

Programmed cell death is an active process that controls cell numbers in several tissues and is involved in morphogenesis during embryonic development and throughout adult life (Thompson, 1995). Cell death can occur by either of two main mechanisms: apoptosis or necrosis (Raff et al., 1993, Schwartzman and Cidlowski, 1993, Williams, 1991). UVR can induce apoptosis by various pathways including DNA damage, mitochondrial damage and direct activation of membrane bound cell death receptors (Pustisek and Situm, 2011, Kulms and Schwarz, 2000).

Studies have shown that PUFAs can regulate cellular proliferation and apoptosis through different mechanisms in vitro and ex vivo (Diep et al., 2000, Sessler and Ntambi, 1998). These include chromatin condensation, phosphatidylserine externalisation, processing and activation of caspases and cleavage of endogenous caspase substrates (Arita et al., 2001, Finstad et al., 1994).

However, the mechanisms used by PUFAs to mediate apoptosis are not fully understood. Some of these apoptotic effects include gene expression, eicosanoid formation, signal transduction and lipid peroxidation (Field et al., 2002). Lipid peroxidation has been found to be associated with increased oxidative stress (Avula and Fernandes, 1999), which is a significant mediator of apoptosis (Buttke and Sandstrom, 1994).

In an animal study, the apoptosis of VSMC cells (vascular smooth muscle cells) was increased when they were treated with DHA. Maximum effect (57%) was shown 24h post (40 μmol/L) DHA treatment. Stimulation of VSMCs with DHA (40 μmol/L) increased both caspase 3 activity and protein expression of active forms of caspase 3 (Diep et al., 2000). Another study using HL-60 cells reported that DNA fragmentation and activation of various caspases were increased when cells were treated with AA and EPA (60μM) and exposed to UVR (Arita et al., 2003).

The effect of n-3 PUFAs on monocytes apoptosis in a human neonatal model was also studied (Sweeney et al., 2007). Here, it was reported that EPA and DHA had dose-dependent effects on monocyte apoptosis. Moreover, AA increased apoptosis in monocytes cells. PUFA concentration ranged from 25 to 100 μmol/L (Sweeney et al., 2007).

Therefore, before we study the protective effect on n-3 PUFA EPA and DHA we must study their possible toxicity and apoptotic effects. The purpose of this study was to evaluate this in the in vitro model system of HaCaT keratinocytes and 46BR.1N fibroblastic cells. The objectives were as follows:

A) Identify an effective UVR dose that does not reduce cell viability by more than 80%.

B) Identify the range of toxic UVR doses that reduce cell viability by more than 50%.

C) Assess the toxicity of various doses of n-3 PUFA.

D) Assess the toxicity of UVR and n-3 PUFA in combination.

E) Investigate the degree of apoptosis induced by n-3 PUFA.

4.2. Materials and Methods

All information relevant to the experiments reported in this study is presented in section 2.2. Cell culture and passaging in section 2.2.3.2 and cell counting in section 2.2.3.2. The effect of UVR exposure on the temperature of the UV box is reported in section 2.2.3.3.1 and exposure of cells to UV radiation in section 2.2.3.3.2. Section 2.2.3.4 describes the treatment of cells with fatty acids and section 2.2.3.5 shows the collection of cells and media. Fatty acid analysis is reported in section 2.2.4.

4.3. Results

4.3.1. Effect of UV exposure on temperature at UV box

This experiment was performed in order to measure the temperature inside the UV box and ensure that it was stable and did not increase during the UV exposure and affect the cell viability. Pre-warmed (37°C) PBS (5ml) and a Thermocouple were used for this measurement. The results show that the PBS temperature did not increase during the UVR exposure. This means that temperature inside the UV box was stable and did not change during the experiment. The results are shown in Table 4.1

Table 4.1. Temperature inside the UV box before (0mJ/cm^2) and during UV irradiation (20-200mJ/cm^2) (n=2 readings of 1 experiment).

UVR (mJ/cm^2)	0	20	40	60	80	100	120	140	160	180	200
Temperature °C	37	37	37	37	36	36	35	35	34	34	34

4.3.2. Effect of skin epidermis layer on UVR intensity

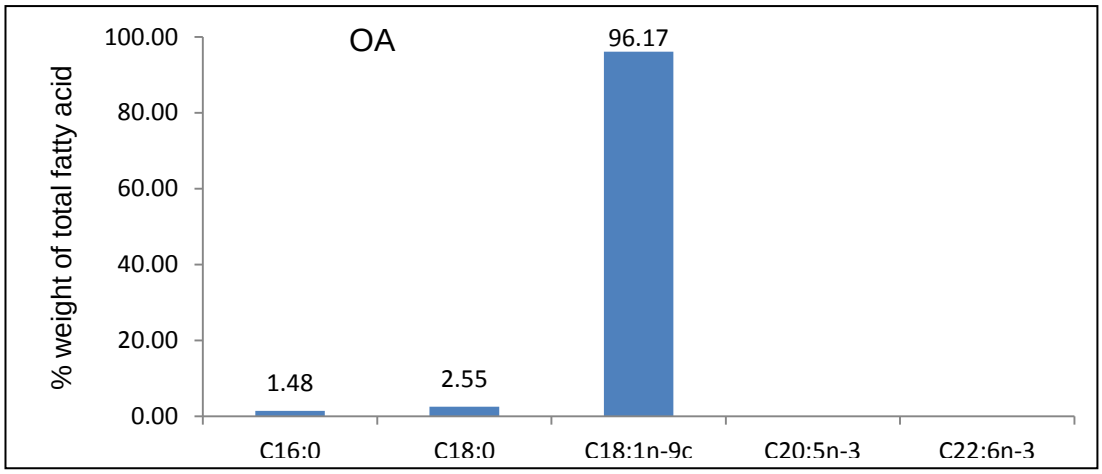
The effect of placing a layer of human skin epidermis in the UV light path was tested using a light meter to measure the intensity (mW/cm^2) of UVR. The results are shown in Table 4.2. The intensity of UVR was 0.33 mW/cm^2 when it was measured without anything placed above the light meter. A similar result was found when clean quartz glass slide was placed above the light meter. However, the intensity decreased to 0.13 mW/cm^2 when a layer of human skin epidermis was placed on the quartz glass slide and both of them placed above the light meter.

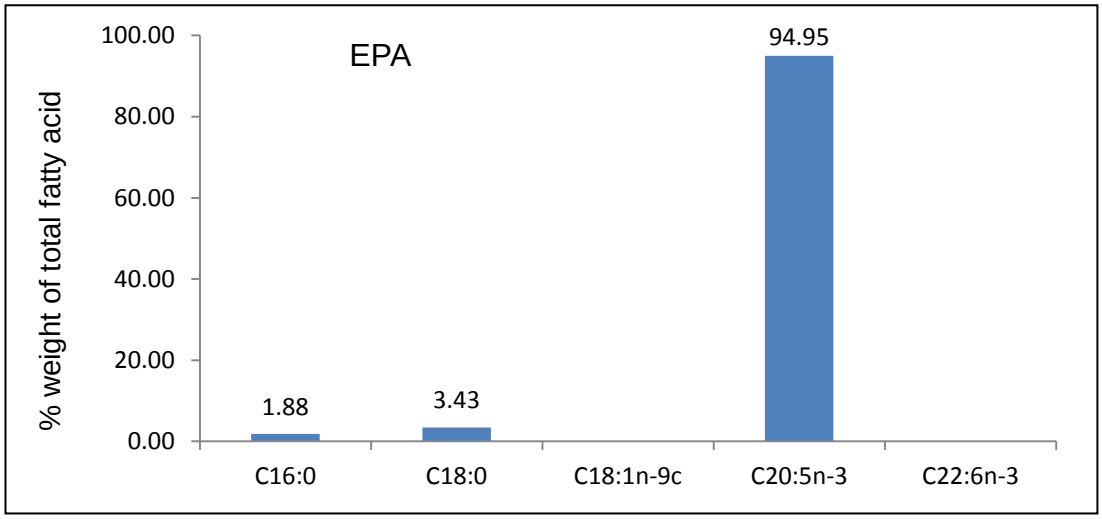
Table 4.2. Effect of placing a layer of skin epidermis in the UV light path

Exposure Time(min)	UVR Intensity (mW/cm^2)		
	(-) Quartz (-) Epidermis	(+) Quartz (-) Epidermis	(+) Quartz (+) Epidermis
1	0.32	0.31	0.13
2	0.31	0.30	0.13
3	0.33	0.30	0.13
4	0.33	0.30	0.13
5	0.33	0.30	0.13
6	0.33	0.30	0.13
7	0.33	0.32	0.13
8	0.33	0.33	0.13
9	0.33	0.33	0.13
10	0.33	0.33	0.13

4.3.3. Analysis of fatty acid stock solution

The fatty acids were prepared as stock solutions of 50 mM in DMSO, aliquoted and stored at –20°C. In order to assess the purity of each stock solution, they were analysed by GC. Results in Figure 4.1 confirmed the identity of these fatty acids and they were not pure. The purity of OA was~ 96%, EPA 95% and 91% was DHA. C16:0 and C18:0 were impurities found in stock solutions and not any other n-3 PUFA.





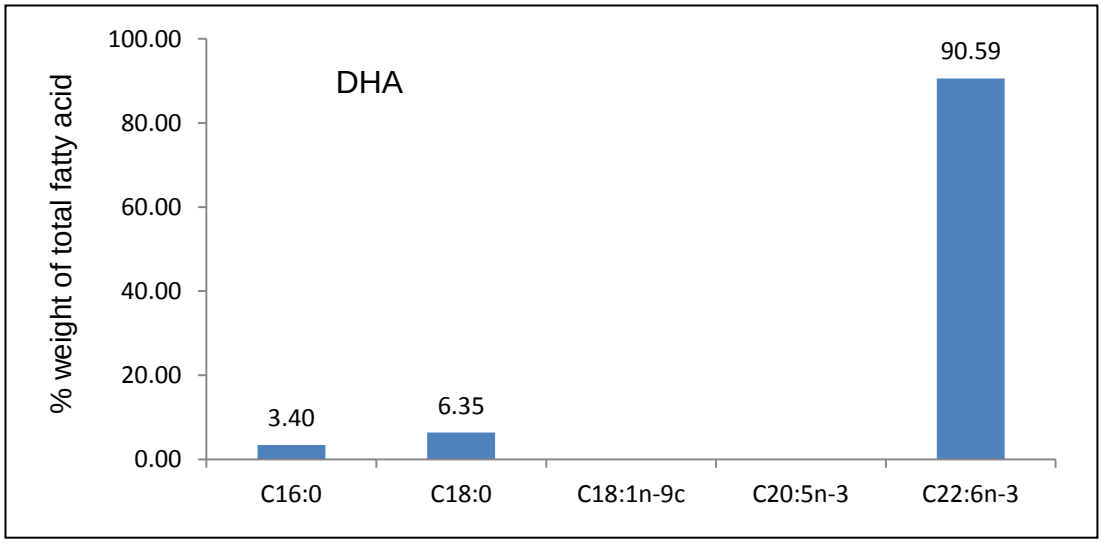


Figure.4.1. GC-FID analysis of oleic acid (OA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) stock solutions (50mM) used in this study to treat the cells. Results are expressed as % weight of total fatty acids of 2 capsules per fatty acid, each analysed in duplicate.

4.3.4. The effect of UVR and fatty acids treatment on HaCaT cell viability and apoptosis

4.3.4.1. Determination of UVR dose

These experiments were performed in order to determine the dose of UVR that results in a) cell viability greater than 80% (effective dose) and b) cell viability of about 50% (toxic dose). The viability was measured using the MTT assay.

4.3.4.1.1. Cell viability at 4h post UVR

Confluent cells were exposed to 20, 60, 90, 100, 200, 300 and 400 mJ /cm^2 and cell viability was measured at 4h post UVR. No significant changes occurred in cell viability between the control and groups which were exposed to UVR doses of 20, 60 and 90 mJ/cm^2 (Figure 4.2). Here, cell viability decreased no more than 95%. However, significant decreases in cell viability occurred when cells were exposed to 100, 200 and 300 mJ /cm^2: viability decreased significantly at the UV dose increased (Figure 4.2). A decrease in cell viability to 80% (effective dose) was noted at 200 mJ /cm^2. Cell viability was reduced to 45% (toxic dose) when cells were treated with 300 mJ /cm^2 (Figure 4.2).

4.3.4.1.2. Cell viability at 24h post UVR

When confluent HaCaT cells were exposed to 15, 30, 70, 100, 130 and 160 mJ/cm^2, the number of viable cells at 24h post UVR decreased compared to the non-irradiated cells (Figure 4.3). The viability of the cells decreased to around 80% when cells were treated with15 mJ/cm^2, so this dose was selected for further studies. Also the toxic dose at 50 mJ/cm^2 (cell viability 50%) selected using the dose study shown in Figure 4.3.

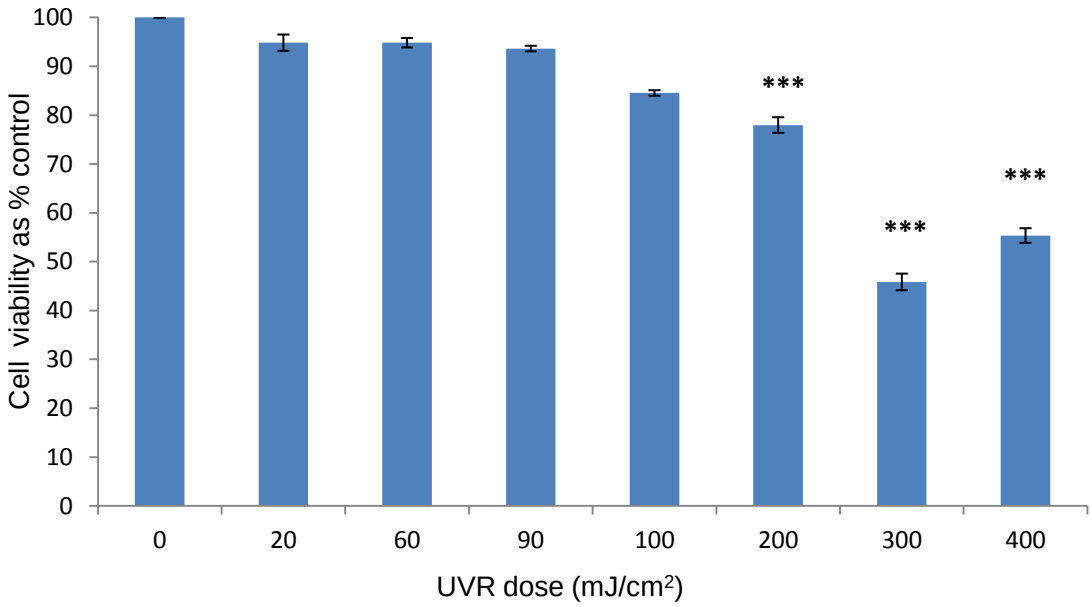


Figure 4.2. Effect of UVR dose on HaCaT keratinocyte viability, assessed 4h post UVR. Results are shown as mean ± SD for 3 independent experiments (each one performed in triplicate). ***p≤0.001, comparing data to untreated control.(0).

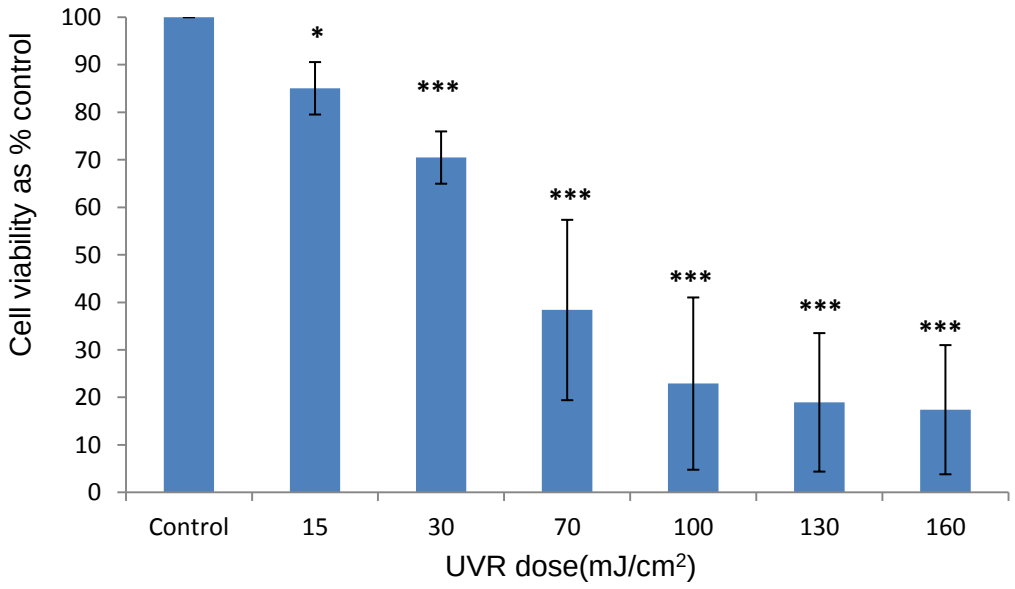


Figure 4.3. Effect of UVR dose on HaCaT keratinocyte viability, assessed 24h post UVR. Results are shown as mean ± SD for 3 independent experiments (each one performed in triplicate). *p≤0.05, ***p≤0.001, comparing data to untreated control.

4.3.4.2. The effect of fatty acid and UVR treatment on HaCaT cell viability

HaCaT cells were treated with OA, EPA and DHA at two doses: 10 and 50 µM. The fatty acids were dissolved in DMSO to prepare stock solutions of 50 mM. The fatty acid solutions were further diluted with culture media to get the desired final concentration. The cells that were not treated with any fatty acids were treated to an equal amount of DMSO during the incubation to serve as controls. After 72h of treatment with fatty acid in complete media, the cells were transferred to PBS and exposed to 15 and 50 mJ/cm^2 of UVR. Free fatty acid complete media was added to the cells and incubated at 37°C. Cell viability was assessed by the MTT assay 24 h post UVR.

4.3.4.2.1. Effect of fatty acid treatment on HaCaT cell viability

Figure 4.4A shows the effect of OA treatment on HaCaT cell viability. There was no significant decrease in cell viability when treated with 10 µM of OA. Cell viability did not decrease significantly when HaCaT cells were treated with 50 µM of OA either (Figure 4.4A).

There was no significant decrease on cell viability when HaCaT were treated with 10 µM of EPA. Cell viability did not significant decrease when HaCaT cells were treated with 50 µM either (Figure 4.4B).

Finally, when cells were treated with 50 µM DHA their viability decreased to 82% ($p \leq 0.01$). However, a low dose of DHA (10 µM) did not affect cell viability (Figure 4.4C).

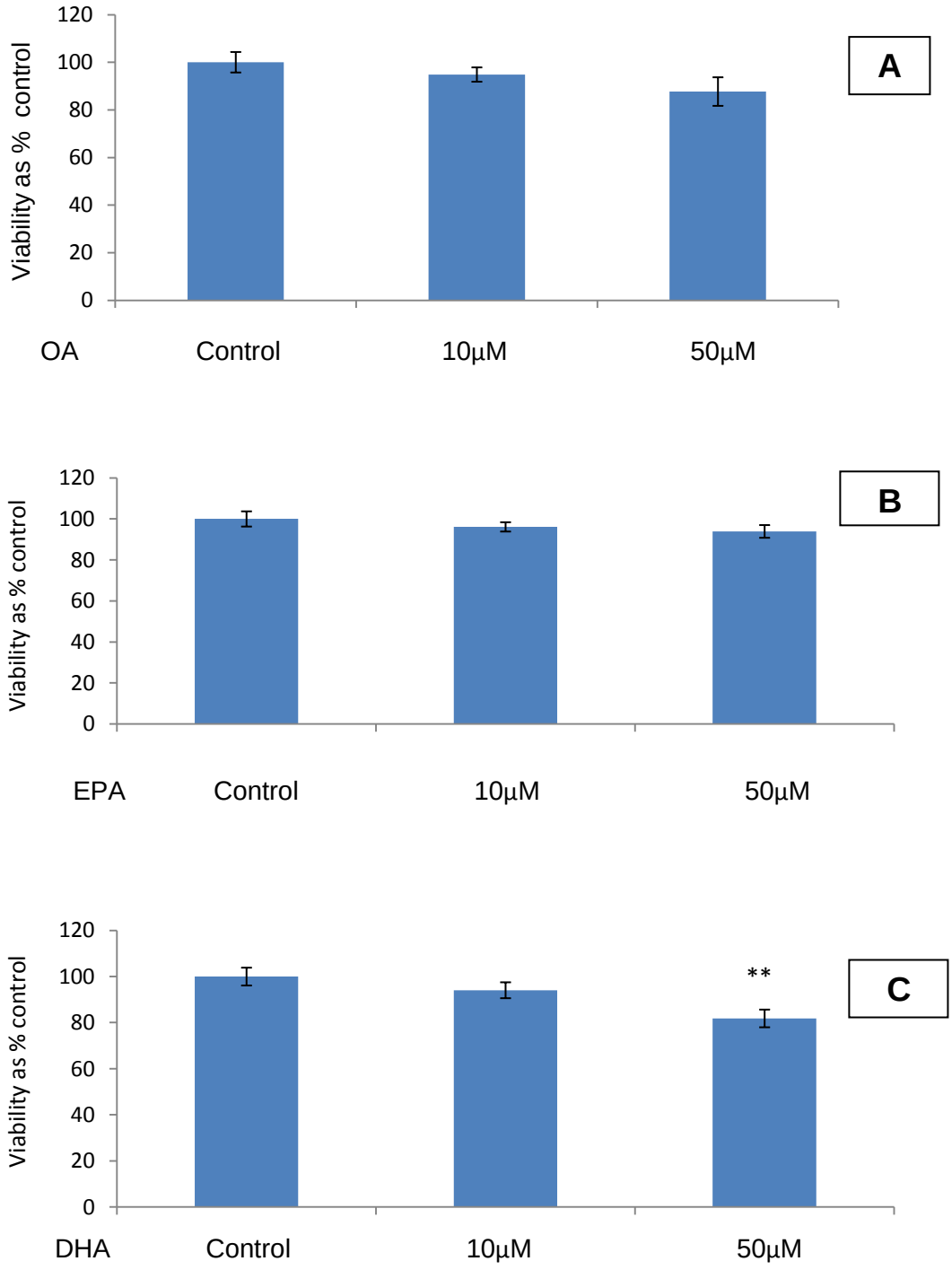


Figure 4.4. The effect of fatty acid treatment, (A) oleic acid (OA), (B) eicosapentaenoic acid (EPA) and (C) docosahexaenoic acid (DHA) on HaCaT keratinocyte cell viability. Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Cell viability was assessed as percent of untreated cells (control). Results are shown as mean ± SD for 3 independent experiments (each one performed in triplicate). **p≤0.01 comparing treatment to control.

4.3.4.2.2. Effect of fatty acid treatment on HaCaT cell viability at 24 h post 15mJ/cm^2 UVR

A decrease in cell viability ($p \leq 0.05$) was observed when HaCaT cells were exposed to 15mJ/cm^2 UVR; this fell to 85% compared to the non- irradiated (FA(-)/UVR(-)) controls (Figure 4.5).

A significant decrease in cell viability was observed when cells treated with 10 µM OA, were exposed to 15 mJ/cm^2 ($p \leq 0.01$). Moreover, the viability of HaCaT cells treated with 50 µM OA showed a further significant decrease to 83% when irradiated with 15 mJ/cm^2 ($p \leq 0.01$) (Figure 4.5A).

EPA-treated cells showed decreased cell viability after UV exposure as shown in Figure 4.5B. Viability of HaCaT cells treated with EPA (10 and 50µM) was significantly decreased to 80% ($p \leq 0.01$) and 77% ($p \leq 0.01$) respectively, when cells were exposed to 15 mJ/cm^2 of UVR and compared to the non-irradiated (FA(-)/UVR(-)) control (Figure 4.5B).

Following exposure to 15 mJ/cm^2, HaCaT cells treated with 10 µM DHA showed a statistically significant decreased cell viability to 80%, when compared with the non- irradiated (FA(-)/UVR(-)) control ($p \leq 0.001$) (Figure 4.5C). Further decrease in cell viability was observed when cells were treated with 50 µM DHA; cell viability was significantly decreased to 67% ($p \leq 0.001$) compared to the non- irradiated (FA(-)/UVR(-)) controls.

Significant decreased on cell viability was observed when HaCaT cell treated with 50µM EPA ($p \leq 0.05$) and DHA ($p \leq 0.001$) and exposed to 15mJ/cm^2 UVR compared to the irradiated cells (FA(-)/UVR(+)) controls (Figure 4.5.B,C).

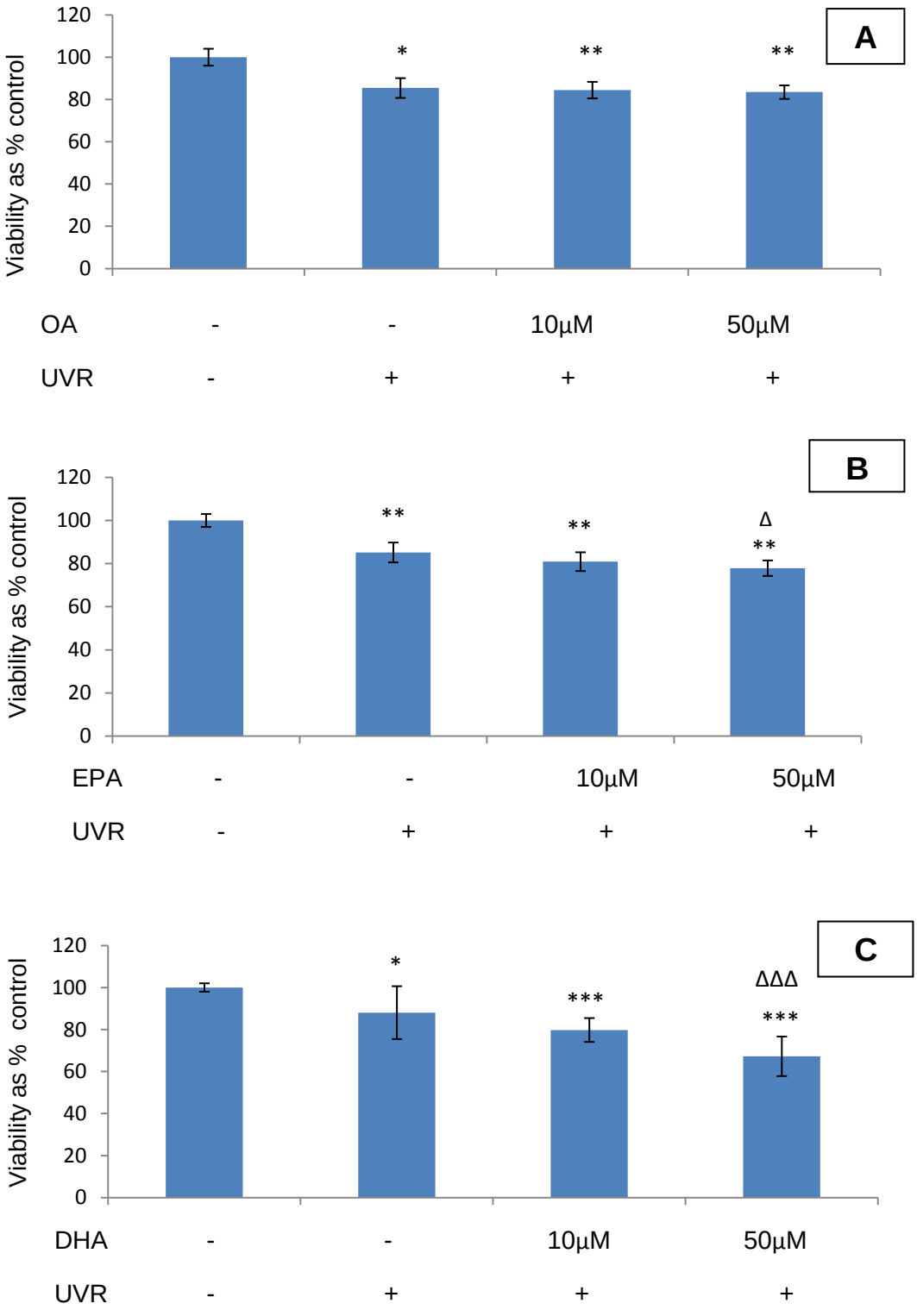


Figure 4.5. The effect of fatty acid treatment, (A) oleic acid (OA), (B) eicosapentaenoic acid (EPA) and (C) docosahexaenoic acid (DHA) on HaCaT keratinocyte cells viability at 24 h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Cell viability was assessed as percent of untreated cells (control). Results are shown as mean ± SD for 3 independent experiments (each one performed in triplicate). *p≤0.05**,ΔΔp≤0.01, ***,ΔΔΔp≤0.001, *=compared to non irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+))

4.3.4.2.3. The affect of fatty acid treatment on HaCaT cell viability at 24 h post 50mJ/cm^2 UVR

A significant decrease in cell viability to approximately 68% occurred when HaCaT cells were exposed to 50mJ/cm^2 UVR ($p \leq 0.01$) (Figure 4.6).

The viability of HaCaT cells treated with 10 and 50 µM of OA was reduced at 64% and 61% respectively, when irradiated with 50 mJ/cm^2, ($p \leq 0.01$, $p \leq 0.01$, respectively) (Figure 4.6A).

The viability of HaCaT cells treated with EPA was also at the same levels as the UVR treated cells, 67% (10µM) and 66% (50µM). This was significantly reduced compared to the untreated control ($p \leq 0.001$, $p \leq 0.001$, respectively) (Figure 4.6B).

When HaCaT cells were treated with DHA and exposed to 50 mJ/cm^2 UVR, their viability was further decreased. This further decreased was statistically significant and reached 55% when cell treated with 10 µM ($p \leq 0.001$) and 46% when cell treated with 50 µM ($p \leq 0.001$) (Figure 4.6C).

Statistically, OA and EPA did not have any effects and there was no difference when compared to irradiated cell (FA(-)/UVR (+)). Viability of HaCaT cells treated with DHA 50µM was significantly decreased ($p \leq 0.05$) when cells were exposed to 50 mJ/cm^2 UVR and compared to the irradiated control (FA(-) / UVR (+)) (Figure 4.6.C).

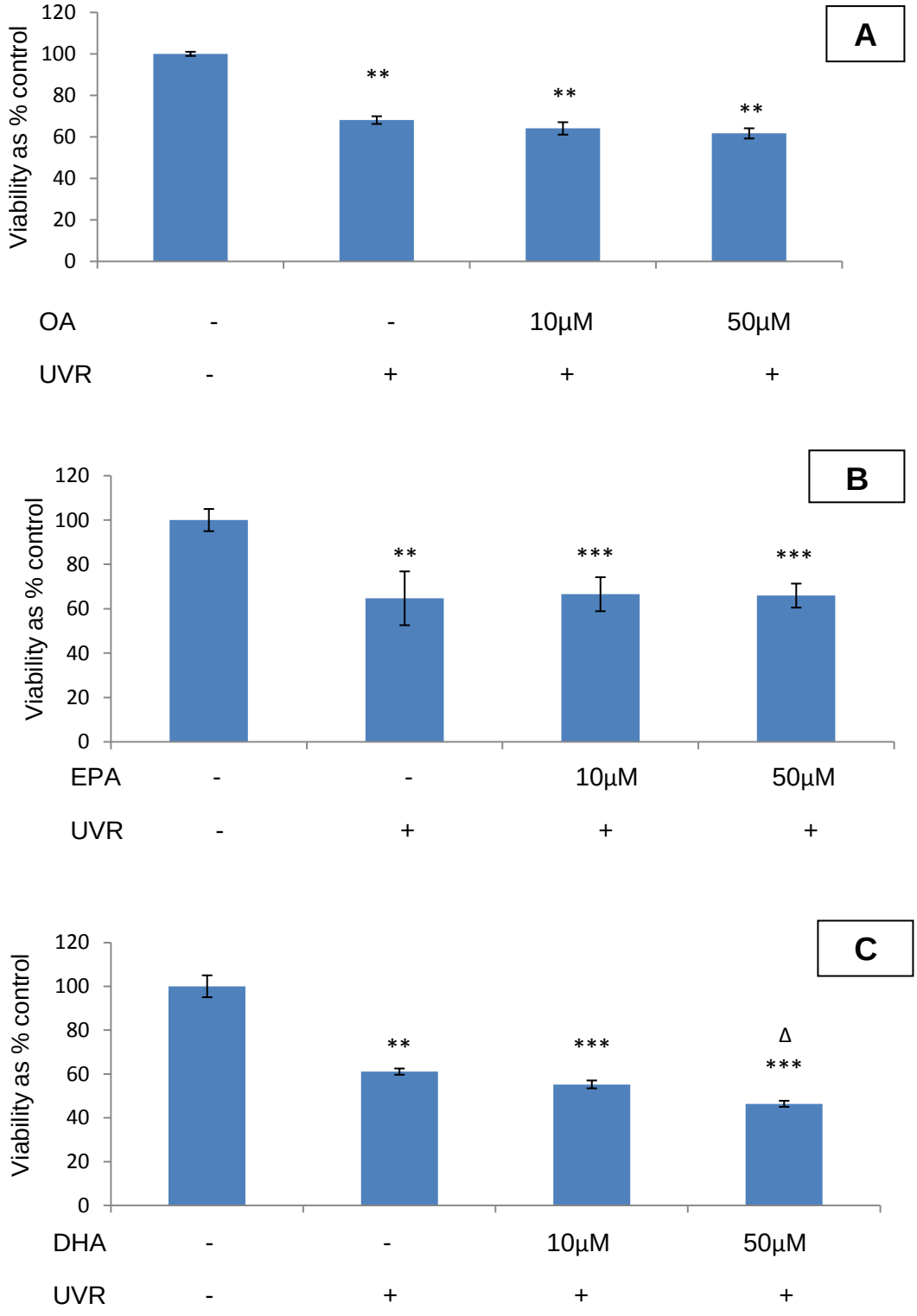


Figure 4.6. Effect of fatty acid treatment, (A) oleic acid (OA), (B) eicosapentaenoic acid (EPA) and (C) docosahexaenoic acid (DHA) on HaCaT keratinocyte cells viability at 24 h post UVR (50 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Cell viability was assessed as percent of untreated cells. Results are shown as mean ± SD for 3 independent experiments (each one performed in triplicate). *,Δp≤0.05,**p≤0.01, ***p≤0.001, *=compared to non irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

4.3.4.3. The affect of fatty acid and UVR treatment on HaCaT cell apoptosis

The apoptosis experiments were performed in order to further explore the previous results that n-3 PUFA reduced viability of the HaCaT cells after exposure to 15 and 50 mJ/cm^2 UVR.

In order to assess the effect of n-3 PUFA in cell apoptosis, HaCaT cells were treated with EPA and DHA (10 and 50 µM) and irradiated with a low and high dose of UVR. Apoptosis was measured using APOPercentage™ kit. During apoptosis, phosphatidyl serine is transferred to the outside of the cell membrane. The Apopercentage dye binds to phosphatidyl serine and the dye is taken up into the cell. The dye in the labelled cells is then released into a lysis solution. Measuring the concentration of dye reflects degree of cell apoptosis. The result was assessed as % of a positive control (H_2O_2). H_2O_2 was prepared to a final concentration of 10mM and cells treated for 4h (100% apoptosis)

4.3.4.3.1. Effect of fatty acid treatment on HaCaT cell apoptosis

Figure 4.7A shows the effect of EPA treatment on HaCaT cell apoptosis. Although there was an increase in the % apoptotic cells when were treated with 10 and 50 µM of EPA, this was not statistically significant.

A low dose of DHA (10 µM) showed no significant increase on cell apoptosis. In contrast when cells were treated with 50 µM DHA for 72 h, cell apoptosis was significantly increased compared to the corresponding controls ($p \leq 0.01$) (Figure 4.7B).

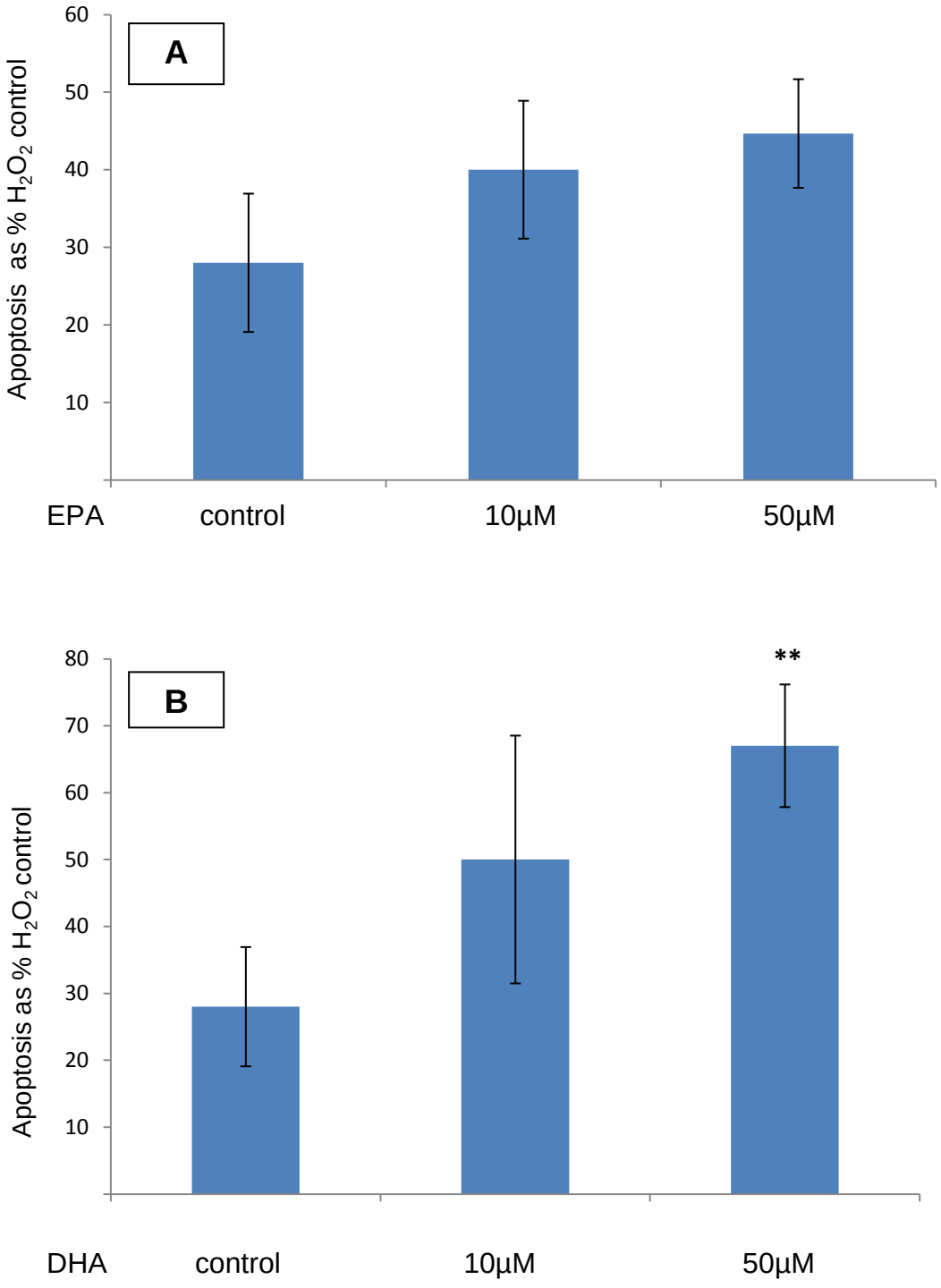


Figure 4.7. The effect of n-3 polyunsaturated fatty acid treatment, (A) eicosapentaenoic acid (EPA) and (B) docosahexaenoic acid (DHA) on HaCaT keratinocyte cells apoptosis. Cells were treated with two concentrations of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (10µM and 50 µM) for 72h. Cell apoptosis was assessed as percent of H_2O_2 (10mM) treated cells (positive control). Results are shown as mean ± SD of 3 independent experiments (each one performed in triplicate). **P≤0.01 compared treatment to control.

4.3.4.3.2. Effect of fatty acid treatment on HaCaT cell apoptosis at 24 h post 15mJ/cm^2 UVR

An increase in cell apoptosis was observed when the cells were exposed to 15mJ/cm^2 UVR, but this did not reach statistical significance (Figure 4.8)

Figure 4.8A shows that a significant increase in cell apoptosis was observed when cells treated with 10 µM EPA and then exposed to 15 mJ/cm^2 ($p \leq 0.05$). Moreover, the apoptosis of HaCaT cells treated with 50 µM EPA appeared to be further increased when irradiated with 15 mJ/cm^2, in comparison to the non-irradiated corresponding control ($p \leq 0.05$) (Figure 4.8A)

The same effect was observed in DHA-treated cells. There was a significant increase on cell apoptosis following exposure to 15mJ/cm^2 UVR and after was treated with 10 µM of DHA ($p \leq 0.05$) or 50 µM of DHA ($p \leq 0.001$) (Figure 4.8B).

In order to see if PUFA have an addition effects on cell apoptosis after UVR, treated and irradiated cell were compared to irradiated cell (FA(-)/UVR (+)). Results in Figure 4.8 shown that low dose (10µm) of EPA and DHA have no significantly effect on cell apoptosis when compared to irradiated cells. HaCaT cell apoptosis was only significantly different when cells treated with 50µM EPA ($p \leq 0.01$) and DHA ($p \leq 0.001$) were exposed to 15mJ/cm^2 UVR comparing to the irradiated corresponding control (FA(-) / UVR (+)).

4.3.4.3.3. Effect of fatty acid treatment on HaCaT cell apoptosis at 24 h post 50 mJ/cm^2 UVR

There was no significant increase of cell apoptosis when un-treated HaCaT cells were exposed to 50mJ/cm^2 UVR (Figure 4.9).The results shown in Figure 4.9A suggest that cell apoptosis was significantly increased ($p \leq 0.05$)

when cells were treated with 10 μM EPA were expose to 50 mJ/cm^2. A further increase in HaCaT cell apoptosis was observed when cells treated with 50 μM EPA were exposed to 50 mJ/cm^2 ($p \leq 0.001$) (Figure 4.9A).

HaCaT cell apoptosis increased when cells treated with DHA were exposed to 50mJ/cm^2 UVR. Specifically, apoptosis significantly increased to 64% ($p \leq 0.01$) when the cells were treated with 10 μM of DHA, and this was even higher when cells were treated with 50μM DHA (95%) ($p \leq 0.001$) (Figure 4.9B).

A significant increase on HaCaT cell apoptosis was observed when cells were treated with EPA 50μM ($p \leq 0.05$), and DHA ($p \leq 0.01$) and exposed to 50 mJ/cm^2 UVR were compared to the irradiated controls (FA (-)/UVR (+))

Overall the apoptosis experiments showed that:

a) UVR increased cell apoptosis but this was not statistically significant.

b) EPA and DHA treatment increased apoptosis in the HaCaT cells and a further statistically significant increase was observed when the treated cells were exposed to 15 or 50 mJ/cm^2. Furthermore, the apoptosis appeared to be fatty acid dose dependent.

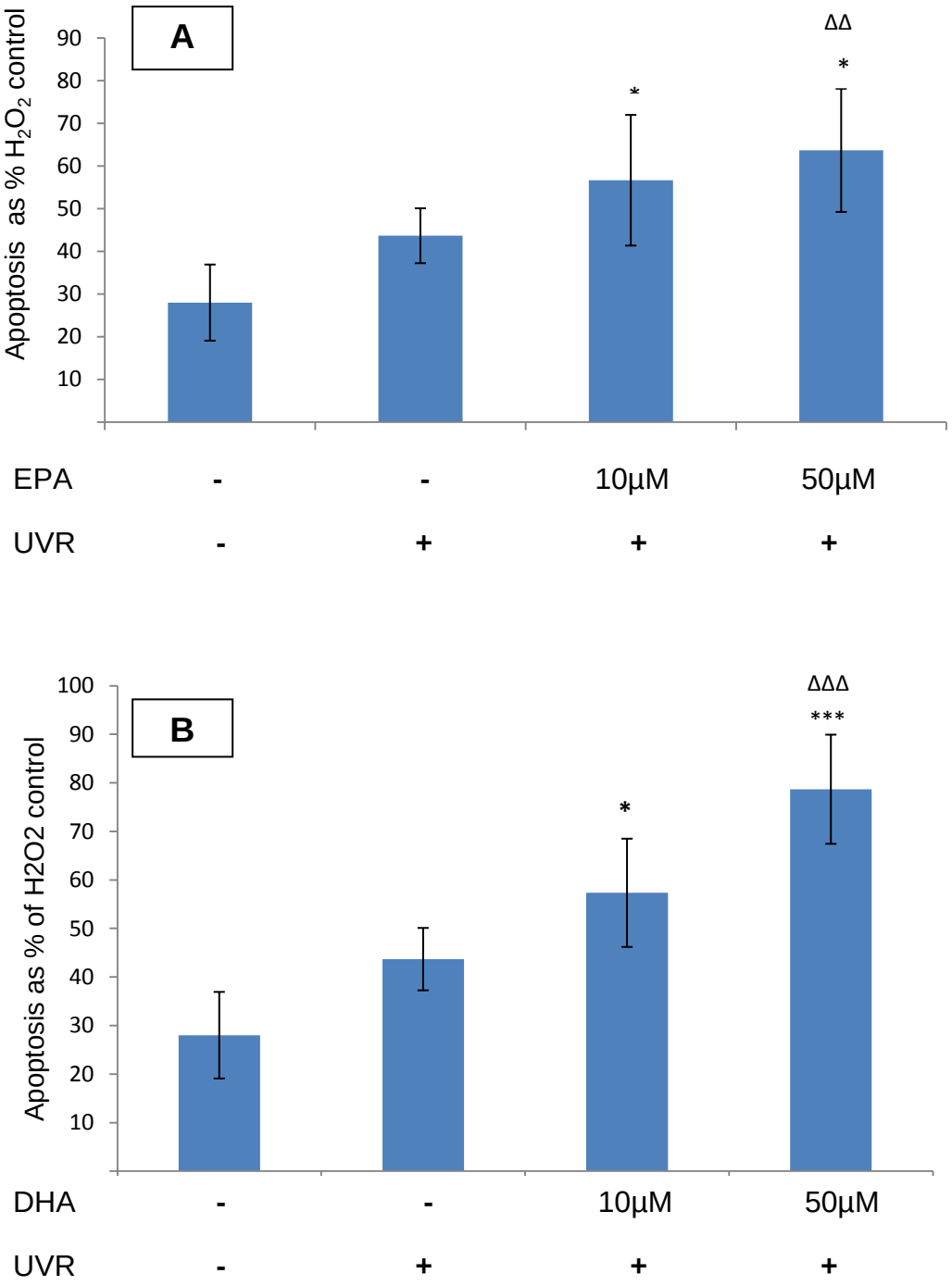


Figure 4.8. The effect of n-3 polyunsaturated fatty acid treatment, (A)eicosapentaenoic acid (EPA) and (B) docosahexaenoic acid (DHA) on HaCaT keratinocyte cells apoptosis at 24 h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Cell apoptosis was assessed as percent of H_2O_2 (10mM) treated cells (positive control). Results are shown as mean ± SD of 3 independent experiments (each one performed in triplicate). *p≤0.05, ΔΔp≤0.01, ***,ΔΔΔp≤0.001, *= comparing to non irradiated control (FA(-)/UVR(-)). Δ=comparing to irradiated control (FA (-)/UVR(+)).

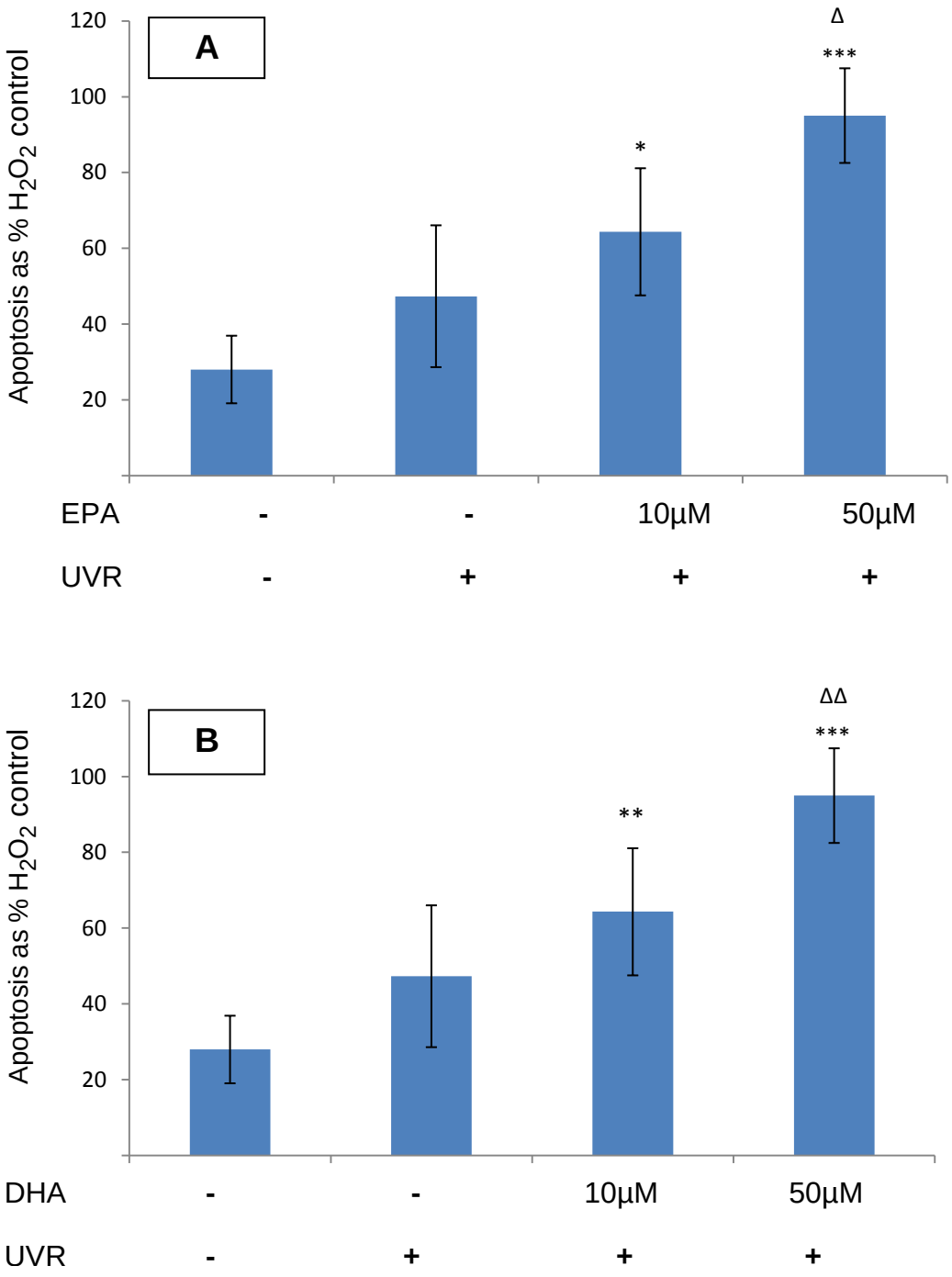


Figure 4.9. The effect of n-3 polyunsaturated fatty acid treatment, (A) eicosapentaenoic acid (EPA) and (B) docosahexaenoic acid (DHA) on HaCaT keratinocyte cells apoptosis at 24 h post UVR (50 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Cell apoptosis was assessed as percent of H_2O_2 (10mM) treated cells (positive control). Results are shown as mean ± SD of 3 independent experiments (each one performed in triplicate). *,Δp≤0.05, ***,ΔΔΔp≤0.001, *= compared to non irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

4.3.4.4. Effect of skin epidermis layer on HaCaT ell viability

In ordered to observe the effect of the epidermal skin layer in protecting cells from UV radiation, HaCaT cells were cultured in two 24 well culture plates. 3 wells for each plate were covered with epidermis and other 3 were not. The plates were placed under UV lamp and exposed for 5 and 10 min. During irradiation the cells were covered with PBS. The HaCaT cells viability was tested directly after UV exposed by MTT assay.

There was a 80% decrease in cell viability when the cells were not covered with the epidermis compared to non irradiated control (Figure 4.10). However, when the cells were covered with the epidermal layer, cell viability was only decreased to approximately 20% of the non irradiated control (Figure 4.10).

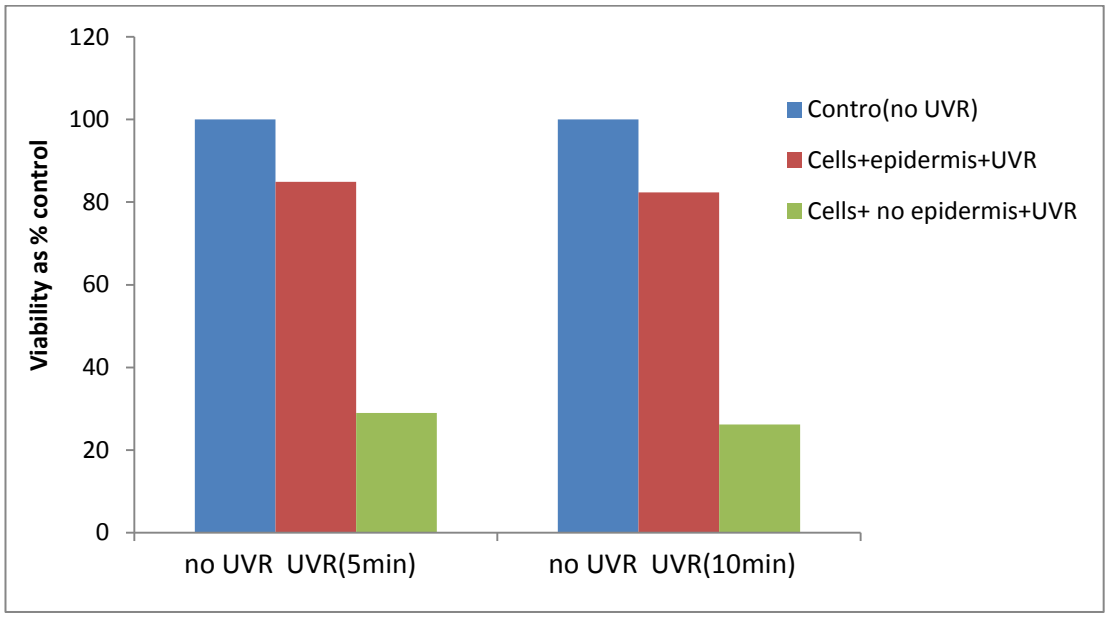


Figure 4.10. HaCaT keratinocyte cell viability with and without the epidermal layer placed directly the UVR. Each condition was performed in triplicate wells. The cell viability was assessed by MTT assay. Results shown as mean of 3 readings, n=1 experiment.

4.3.5. The effect of fatty acid and UVR treatment on 46 BR.1N cell viability and apoptosis

4.3.5.1. Determination of UVR dose

These experiments were performed in order to determine the dose of UV that results in a) cell viability greater than 80% (effective dose) and b) cell viability of about 50% (toxic dose). The viability was measured using the MTT assay.

4.3.5.1.1. Cell viability at 4h post UVR

Confluent cells were exposed to 15, 50 and 100 mJ /cm^2. Cell viability measured at 4h post UVR was not statistically different between the control (untreated cells) and cells treated with 15 and 50 mJ/cm^2 (Figure 4.11). Cell viability decreased from 100% for the control group to approximately 82% when cells were exposed to 100 mJ /cm^2. This decrease was statistically significant ($p \leq 0.001$) (Figure 4.11).

4.3.5.1.2. Cell viability at 24h post UVR

In order to assess the effect of UVR on 46BR.1N on cell viability, cells were exposed to different UVR doses: 0, 15, 30, 50, 70,100,130 and 160 mJ/cm^2. The results shown that the effect of UVR in cell viability was dose-dependent. The viability was decreased following increased UVR. Cell viability was significantly decreased to 85% ($p \leq 0.01$) at 15mJ/cm^2 compared to un-irradiated control (Figure 4.12).

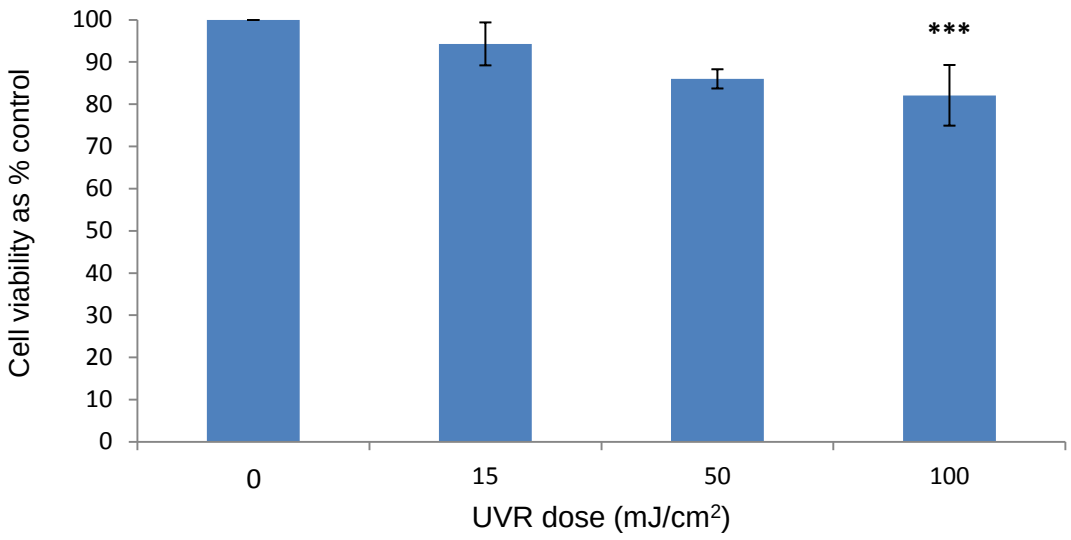


Figure 4.11 Effect of UVR dose on 46BR.1N fibroblast cell viability, assessed 4h post UVR. Results are shown as mean ± SD for 3 independent experiments (each one performed in triplicate). ***p≤0.001, comparing data to untreated control.

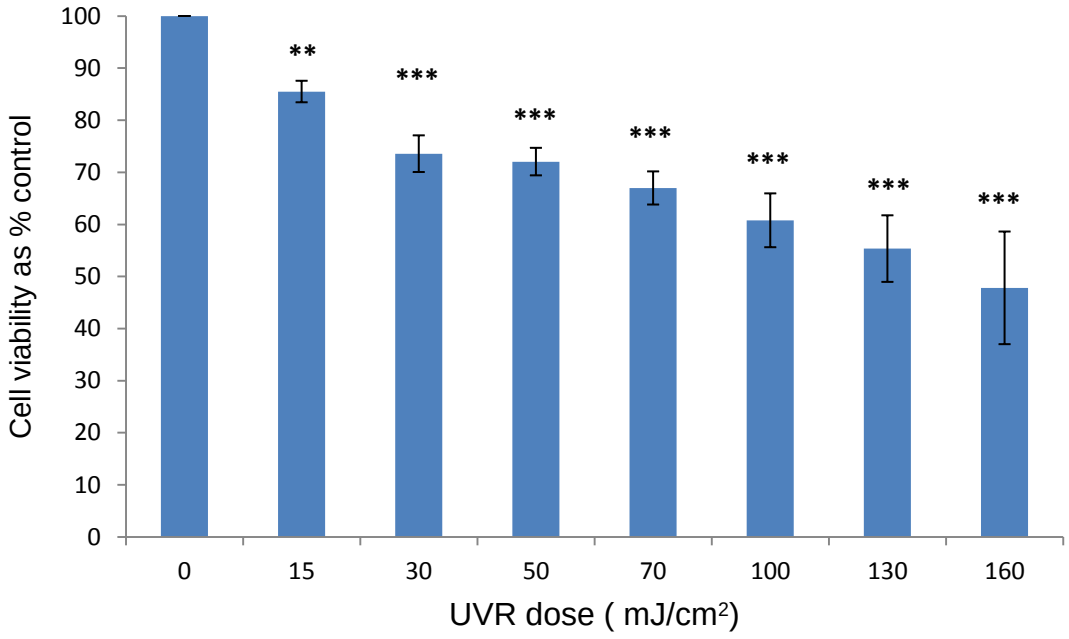


Figure 4.12. Effect of UVR dose on 46BR.1N fibroblast cell viability, assessed 24h post UVR. Results are shown as mean ± SD for 3 independent experiments (each one performed in triplicate). **p≤0.01, ***p≤0.001 comparing data to untreated control.

4.3.5.2. The effect of fatty acid and UVR treatment on 46BR.1N cell viability

Stock solutions of 50 mM of OA, EPA and DHA were prepared in DMSO. The fatty acid solutions were further diluted with culture media to get the desired final concentration (10 and 50 µM). 46BR.1N cells were incubated 72h with different fatty acid treatments in complete media. The cells that were not treated with any fatty acids were treated to an equal amount of DMSO during the incubation to serve as vehicle controls. Cell viability was assessed by the MTT assay 24 h post 15 and 50 mJ/cm^2 UVR.

4.3.4.4.1. Effect of fatty acid treatment on 46BR.1N cell viability

The results in Figure 4.13A show that 46BR.1N viability decreased to 93% and 89% when OA treatment was increased from 10 µM to 50µM respectively. However this was not statistically significant.

Also there was no significant decrease in cell viability when 46BR.1N cells were treated with EPA 10 and 50 µM. Results are shown in Figure 4.13B.

Cell viability did not reach statistical difference when cells were treated with 10 µM DHA. In contrast, a decrease in 46BR.1N cell viability to 73% was observed when the cells were treated with 50 µM DHA; this was statistically significant compared to untreated cells ($p \leq 0.01$). Results shown in Figure 4.13C.

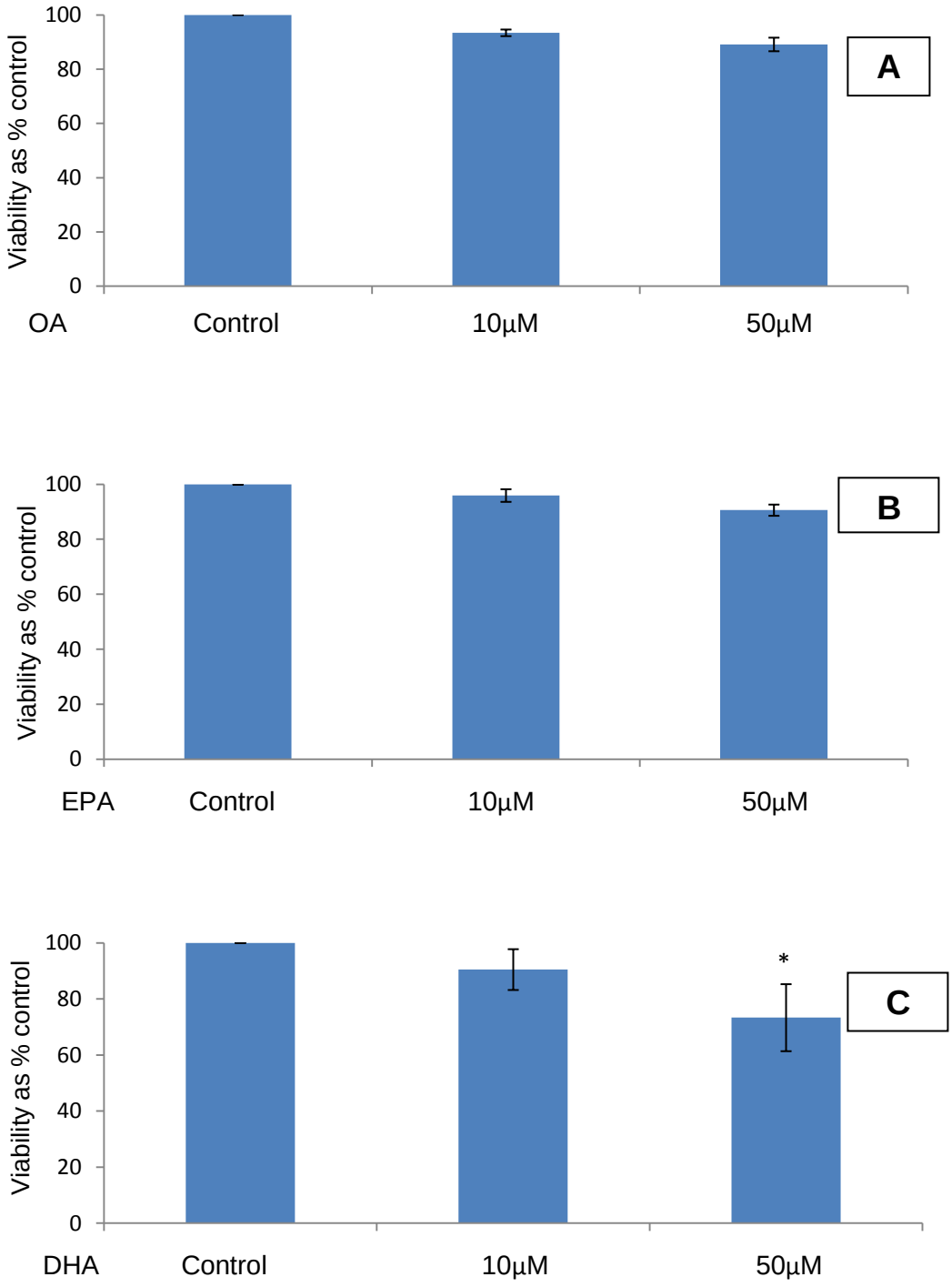


Figure 4.13. The effect of fatty acid treatment, (A) oleic acid (OA), (B) eicosapentaenoic acid (EPA) and (C) docosahexaenoic acid (DHA) on 46BR.1N fibroblast cells viability. Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Cell viability was assessed as percent of untreated cells (control). Results are shown as mean ± SD for 3 independent experiments (each one performed in triplicate). *p≤0.05 comparing treatment to control.

4.3.5.3. The effect of fatty acid treatment on 46BR.1N cell viability at 24 h post 15mJ/cm^2 UVR

Exposure of untreated 46BR.1N cells to 15mJ/cm^2 showed significant decrease in cell viability (86%, $p \leq 0.05$) when compared to the corresponding controls. The dose 15mJ/cm^2 was considered as the active dose and was used in this study (Figure 4.14).

When OA treated cells (10 and 50µM) were exposed to 15mJ/cm^2, Cell viability significant decreased to 82% ($p \leq 0.01$) and 77% ($p \leq 0.01$), respectively. The results are shown in Figure 4.14A.

The same result was observed when cells were treated with EPA; the viability decreased to 83% ($p \leq 0.05$) and 79% ($p \leq 0.01$) when the EPA dose increased from 10 to 50 µM, Figure 4.14B.

As shown in Figure 4.14C, DHA induced a dose-dependent reduction in 46BR.1N cell viability. DHA (10 µM) significantly reduced the viability to 76% ($p \leq 0.01$) compared to control cells. The 50 µM of DHA treatment reduced cell viability even more and this was significantly decreased to 58% ($p \leq 0.001$) compared to control cells.

There was no statistical difference in cell viability between 46BR.1N cells treated with OA and EPA 10 or 50µM, and 10 µM DHA when exposed to 15mJ/cm^2 and compared to the corresponding irradiated controls (FA(-)/UVR(+)). In contrast, when cells were treated with DHA 50µM, DHA showed an additive effect and the cell viability was significantly decreased ($P \leq 0.001$) compared to corresponding irradiated controls (FA (-)/UVR(+)) (Figure 4.14C).

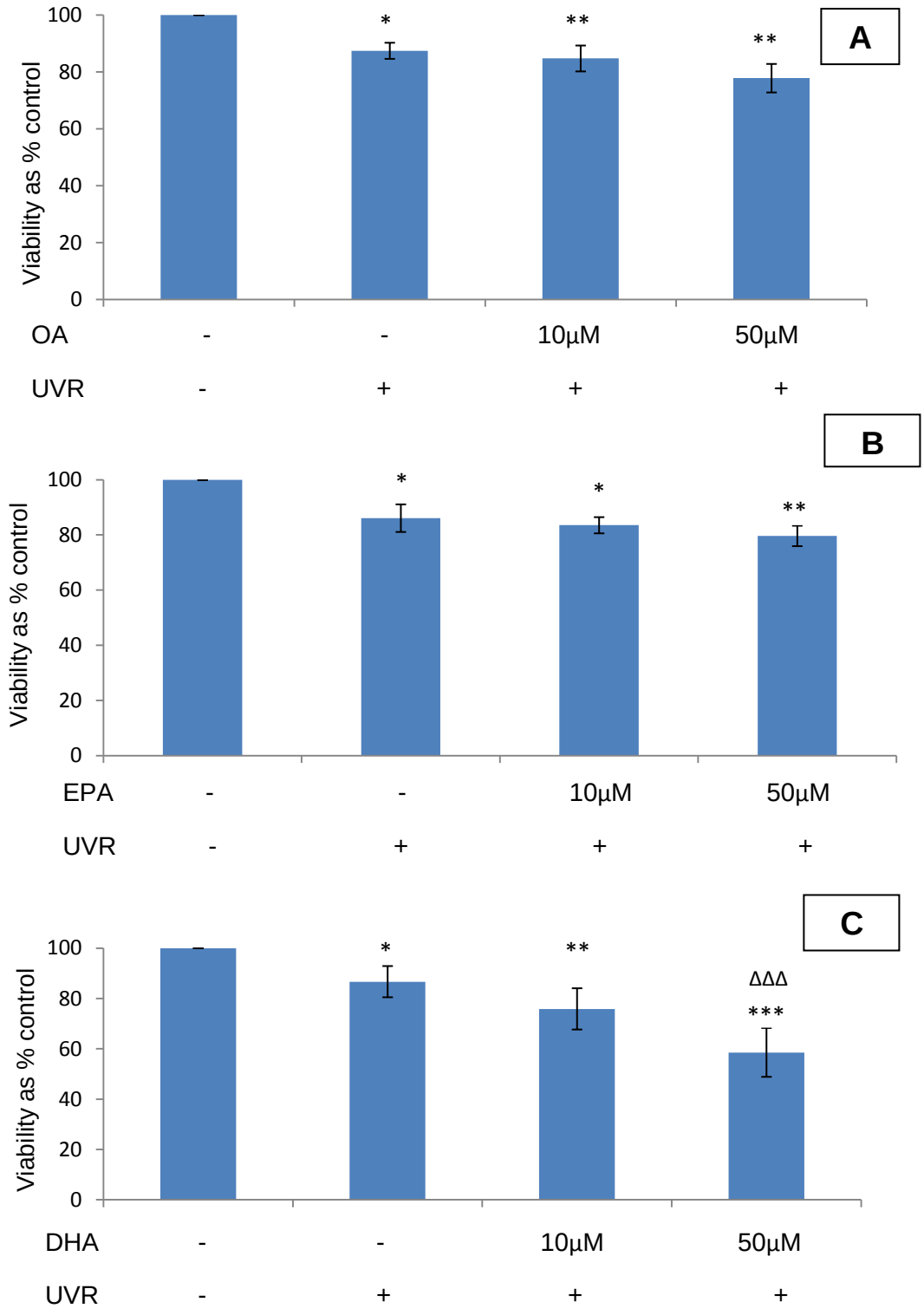


Figure 4.14. Effect of fatty acid treatment, (A) oleic acid (OA), (B) eicosapentaenoic acid (EPA) and (C) docosahexaenoic acid (DHA) on 46BR.1N fibroblast cells viability at 24 h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Cell viability was assessed as percent of untreated cells. Results are shown as mean ± SD for 3 independent experiments (each one performed in triplicate). *p≤0.05,**=p≤0.01, ***,ΔΔΔp≤0.001, *=comparing to non irradiated control (FA(-)/UVR(-)). Δ=comparing to irradiated control (FA (-)/UVR(+)).

4.3.5.4. The effect of fatty acid treatment on 46BR.1N cell viability at 24 h post 50mJ/cm^2 UVR

In order to assess the effect of UVR in 46BR.1N cell viability, the cells were exposed to 50mJ/cm^2 UVR. This was considered to be a toxic dose. The results shown in Figure 4.15 suggest that the viability of 46BR.1N cells was significantly decreased to 65% ($p \leq 0.01$) compare to the corresponding untreated controls.

The effect of OA on 46BR.1N cell viability is shown in Figure 4.15A. It is clear that post 50mJ/cm^2 UVR treatment, cell viability significantly decreased when the dose of fatty acid increased. Cell viability in cells treated with OA (10 and 50µM) was significantly decreased to 61% and 58%, respectively ($p \leq 0.001$and $p \leq 0.001$, respectively), when compared to non treated cells.

The viability of 46BR.1N which were treated with EPA (10 and 50µM) and exposed to 50mJ/cm^2, was significantly decreased to 64% and 60%, respectively ($p \leq 0.001$and $p \leq 0.001$, respectively), when compared to non treated cells as shown in Figure 4.15B.

Cell viability was even lower when cells that were treated with DHA were exposed to 50mJ/cm^2 UVR. Viability was significantly decreased to 64% ($P \leq 0.001$), following 10 µM DHA and 47% ($P \leq 0.001$) following treatment with 50 µM DHA (Figure 4.15C).

OA and EPA did not have any additive effect and when compared to irradiated cells there was no statistically difference. However, 50 µM DHA showed an additive effect to UVR and was significantly different ($P \leq 0.001$) compared to corresponding controls (Control:-/+ fatty acid/UVR) (Figure 4.15C).

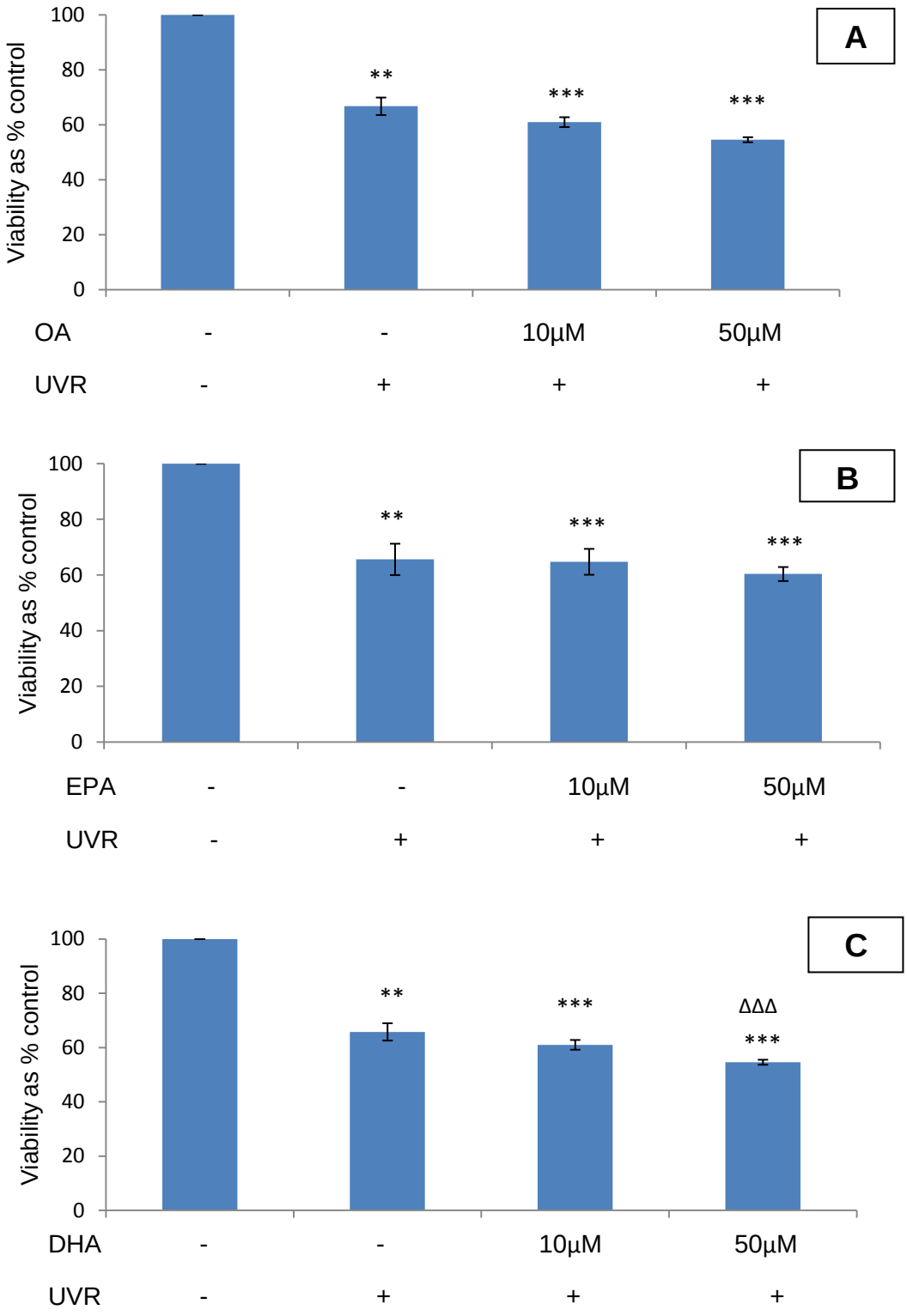


Figure 4.15. Effect of fatty acid treatment, (A) oleic acid (OA), (B) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on 46BR.1N fibroblast cells viability at 24 h post UVR (50 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Cell viability was assessed as percent of untreated cells. Results are shown as mean ± SD for 3 independent experiments (each one performed in triplicate). *p≤0.05,**p≤0.01, ***,ΔΔΔp≤0.001, *=comparing to non irradiated control (FA(-)/UVR(-)). Δ=comparing to irradiated control (FA (-)/UVR(+)).

4.3.5.5. The effect of fatty acid and UVR treatment on 46BR.1N cell apoptosis.

In order to assess the effect of n-3 PUFA and UVR in 46BR.1N apoptosis, the cells were treated for 72h with and without EPA and DHA 10 and 50µM. After that, cells were exposed to 15 or 50mJ/cm^2 UVR, and cells apoptosis was measured at 24h post UVR by using the APOPercentage kit.

4.3.5.5.1. Effect of fatty acid treatment on HaCaT cell apoptosis

Cell apoptosis increased but was not statistically significant when cells were treated with 10 µM EPA. However, a statistically significant increased was observed when the cells were treated with 50 µM EPA ($p \leq 0.01$) compared to untreated cells (FA(-)/UVR(-)) (Figure 4.16A).

Result in figure 4.16B shown that DHA treatment induced apoptosis at 10 µM ($p \leq 0.05$) and 50 µM ($p \leq 0.001$) compared to untreated cells (FA (-)/UVR(-)).

4.3.5.5.2. The affect of fatty acid treatment on 46BR.1N cell apoptosis at 24 h post 15mJ/cm^2 UVR

An increase in cell apoptosis was observed when untreated cells were exposed to 15mJ/cm^2 UVR, but this increase was not statistically significant when compared to non-irradiated group (Figure 4.17).

The apoptosis of 46BR.1N cells treated with 10 and 50µM EPA was significant increase after 15mJ/cm^2 UVR ($p \leq 0.01$ and $p \leq 0.01$) compared to the corresponding non-irradiated controls (Figure 4.17A).

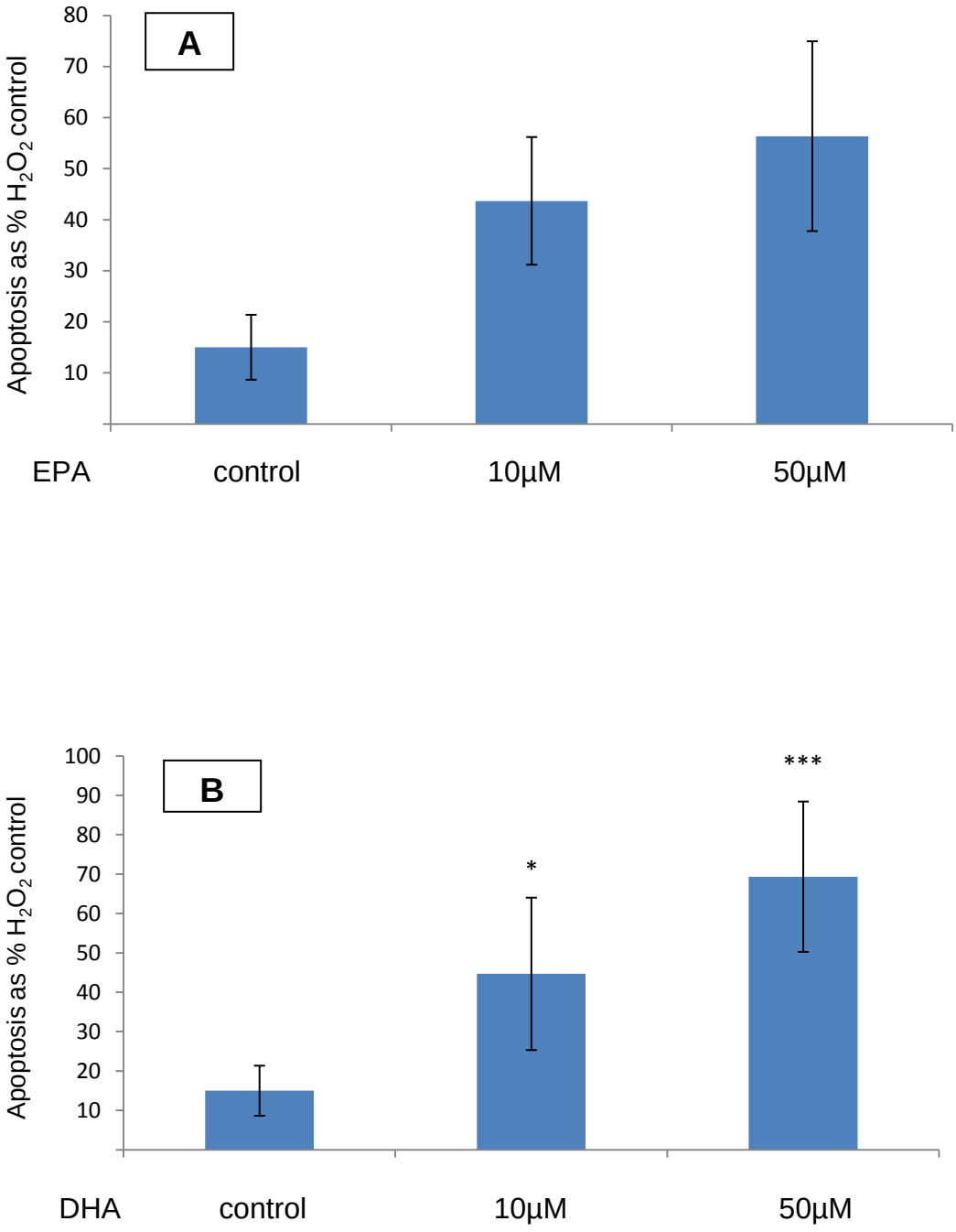


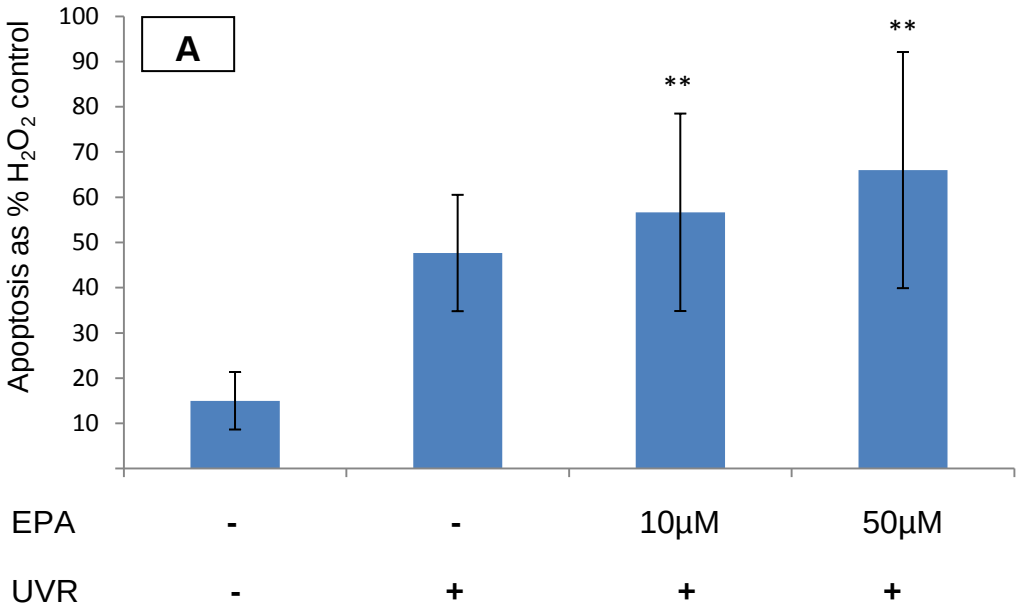
Figure 4.16. The effect of n-3 polyunsaturated fatty acid treatment, (A) eicosapentaenoic acid (EPA) and (B) docosahexaenoic acid (DHA) on 46BR.1N fibroblast cells apoptosis. Cells were treated with two concentrations of EPA and DHA (10µM and 50 µM) for 72h. Cell apoptosis was assessed as percent of H_2O_2 treated cells (positive control) Results are shown as mean ± SD of 3 independent experiments (each one performed in triplicate). *p≤0.05, ***p≤0.001compared treatment to control.

Following exposure to 15 mJ/cm^2, 46BR.1N cells treated with 10 µM DHA showed increase in cell apoptosis, this was statistical significant when compared with the corresponding non-irradiated controls ($p \leq 0.01$) (Figure 4.14.B). Further increase in cell apoptosis was observed when cells were treated with 50 µM DHA. This increase was significant compared to the non-irradiated (FA(-)/UVR(-)) corresponding controls ($p \leq 0.001$) (Figure 4.16B). Also, 50 µM DHA showed an additive effect ($p \leq 0.001$) compared to the irradiated corresponding controls (Figure 4.17B)

4.3.5.5.3. The effect of fatty acid treatment on 46BR.1N cell apoptosis at 24 h post 50 mJ/cm^2 UVR

The apoptosis of 46BR.1N cells was significantly increased (50%) when cells exposed to 50mJ/cm^2 UVR compared to the corresponding non-irradiated controls ($p \leq 0.05$) (Figure 4.18). EPA-treated cells showed an increase on cell apoptosis after UV exposure as shown in Figure 4.18A. Apoptosis of 46BR.1N cells treated with 10 and 50µM EPA was significantly increased to 62% ($p \leq 0.001$) and 72% ($p \leq 0.001$), respectively, when cells were exposed to 50 mJ/cm^2 of UVR and compared to the corresponding non-irradiated control

The results shown in figure 4.18B suggest that, cell apoptosis was significantly increase to 63% when cells were treated with 10 µM DHA and exposed to 50 mJ/cm^2 ($p \leq 0.001$). The apoptosis of 46BR.1N treated with 50 µM DHA and exposed to 50 mJ/cm^2 was further increased to 113% compared to the non-irradiated control (FA(-)/UVR(+) ($p \leq 0.001$) (Figure 4.17.B). Also, 50 µM DHA showed additive effect ($p \leq 0.001$) compared to the irradiated corresponding controls (Figure 4.18B)



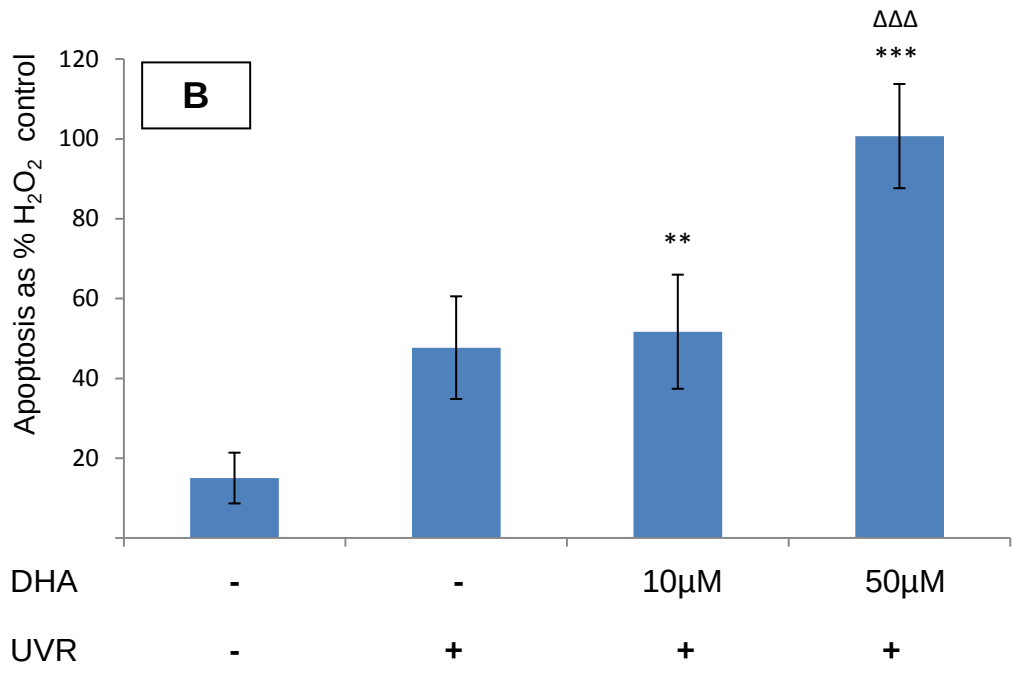


Figure 4.17. Effect of n-3 polyunsaturated fatty acid treatment, (A) eicosapentaenoic acid (EPA) and (B) docosahexaenoic acid (DHA) on 46BR.1N fibroblast cells apoptosis at 24 h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Cell apoptosis was assessed as percent of H_2O_2 (10mM) treated cells (positive control). Results are shown as mean ± SD for 3 independent experiments (each one performed in triplicate). **p≤0.01, ***,ΔΔΔp≤0.001, *=comparing to non irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

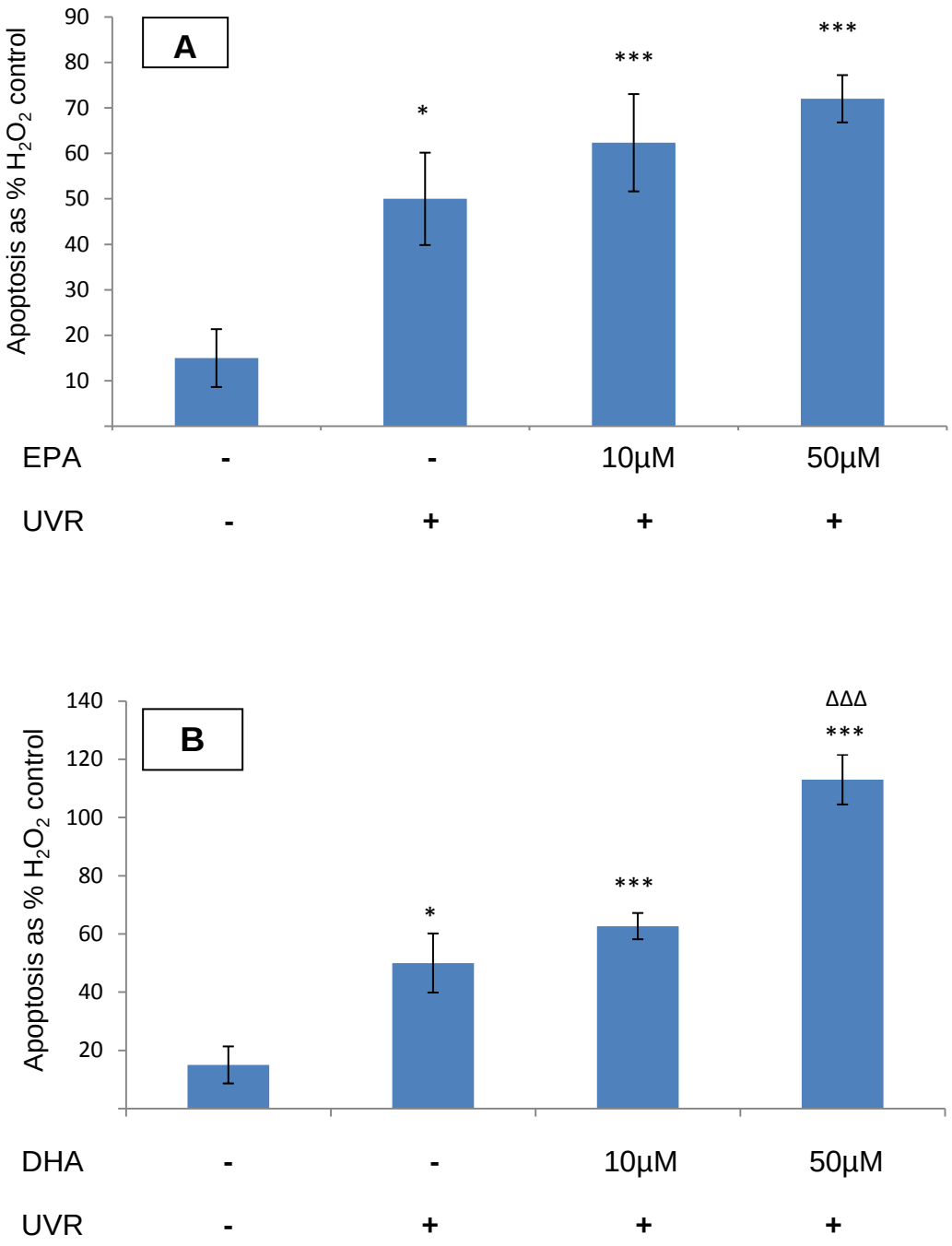


Figure 4.18. Effect of n-3 polyunsaturated fatty acid treatment, (A) eicosapentaenoic acid (EPA) and (B) docosahexaenoic acid (DHA) on 46BR.1N fibroblast cells apoptosis at 24 h post UVR (50 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Cell apoptosis was assessed as percent of H_2O_2 (10mM) treated cells (positive control). Results are shown as mean ± SD of 3 independent experiments (each one performed in triplicate). *=p≤0.05, ***,ΔΔΔp≤0.001, *=compared to non irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

4.4. Discussion

Ultraviolet radiation is an important pathogenic component of solar light. It is one of the most important acute and chronic skin stressors, and has been implicated in inflammation, skin aging and cancer. However, it also has beneficial health effects such as vitamin D3 synthesis.

To assess the effect of UVR on human skin cells, two cell lines one as epidermal keratinocytes (HaCaT) and one as dermal fibroblasts (46BR.1N) were used as models. First, the temperature inside the UV box was measured, because any increase could impact on cell viability. The temperature inside the UV box was measured for a period of time corresponding to exposure of 0 to 200 mJ/cm^2. The temperature was stable during the exposure time (Table 4.1), suggesting that the experiments were not affected by temperature-related factors. The cytotoxic effect of temperature has been investigated with various cutaneous cell lines, such as the human melanoma cell line A375, HaCaT keratinocytes, the epidermoid carcinoma A431, the primary human melanoma cell line WM35, and a metastatic melanoma cell line, HS294T (Shellman et al., 2008). It has been found that various cytotoxic effects appear above 37°C. In another study it was reported that four human cell lines, the human colon carcinoma cell line WiDr, human glioblastoma-astrocytoma U-87MG, human lung carcinoma A-549, and normal human lung fibroblasts CCD-ISLu were relatively resistant to temperatures up to 43°C or 45°C (Armour et al., 1993). Temperatures of more than 43°C were beneficial in patients with superficial tumours receiving radiation therapy (Jones et al., 2005). Human WiDr cells treated with 5-aminolevulinic acid (0.5 mM) and exposed to different temperatures before or after light exposure were found to have decreased

survival chances when exposed to 41 °C for 1 h before light exposure (Juzeniene et al., 2006).

To find a UVR dose that keeps the cell viability greater than 80%, the effect of various doses of UVR were investigated on HaCaT and 46BR.1N cell viability, at 4h and 24h after exposure. After 4h of irradiation HaCaT cells showed a decrease in viability when exposed to doses of 100 mJ/cm^2 and above compared to the non-irradiated cells. However, different results were found when cell viability was measured at 24 h after exposure. Cell viability decreased in HaCaT and 46BR.1N cells exposed to 15, 30, 70, 100, 130 and 160 mJ/cm^2 compared to the non-irradiated cells and at 24 h after exposure. The UVB doses used in our study were 15 and 50 mJ/cm^2. 24h post UVR was chosen as a period after exposure to UVR, as covered all phases of the cell cycle. Relevant to that, one MED is the level of UVR that causes skin erythema (sunburn) which appears as reddening on the skin after 24h. However, in human skin the epidermal layer reduced the level of irradiation reaching the epidermis as shown by our data and reported in the literature (Table 4.2).

In the resent study, the data clearly showed that HaCaT cell viability decreased with increased UV radiation (Figure 4.3). This result was consistent with evidence from the literature. It has been reported that there was no significant decrease in the viability of normal human keratinocytes following UV doses ranging from 4.5 to 36 mJ/cm^2 and at 1h after exposure, but there was a significant decrease at 24h after exposure to 9 mJ/cm^2 (Wong et al., 2000). A decrease in the proliferation of HaCaT cells by 50% was reported following UV exposure to 200 mJ/cm^2 (Park and Lee, 2008). Another study using HaCaT cells reported that there was no effect on cell viability at 48h post UVB doses of

25, 50 or 75 mJ/cm^2, but there was a significant decrease to 86.7% after an exposure to 100 mJ/cm^2 (Storey et al., 2007). HaCaT cell viability was also found to have decreased from 80% to 60% when UVB irradiation increased from 30 to 60mJ/cm^2, respectively; whilst viability was less than 30% when the UVB was 120mJ/cm^2 (Lee et al., 2003).

The effect of UVB on primary human keratinocytes was examined in another study where cell viability measured at 24 h post 5, 10 and 25mJ/cm^2 broadband UVB (BBUVB) significantly decreased (Cho et al., 2008). Lawley et al. (2000) reported that the viability of primary human keratinocytes exposed to 40 and 80mJ/cm^2 UVB was slightly decreased after 3h of UV treatment. A significant decrease in viability was observed at 20h post UV treatment (Lawley et al., 2000). Finally, the viability of a cell line of normal human epidermal keratinocytes was decreased to 95%, 83% and 77% when the cells were exposed to 10, 30 and 60 mJ/cm^2, respectively. Viability was assessed 24h post UVR (Izykowska et al., 2009).

A similar effect was found with fibroblasts 46BR.1N. The cell viability was decreased to less than 80% when cells were exposed to more than 15mJ/cm^2 (Figure 4.11). This was consistent with reports showing that viability of cultured dermal fibroblasts was decreased to 91%, 81%and 56% when exposed to 35, 70, and 140 mJ/cm^2 at 24h post UVR respectively (Ryoo et al., 2001). 100mJ/cm^2 was found to be a toxic dose for cultured normal human dermal fibroblasts extracted from breast skin (Yan et al., 2010). Meanwhil, Storey et al. reported that the viability of dermal fibroblast cells CCD922SK was unchanged after 25 mJ/cm^2 but was reduced to 50% or less when exposed to 50mJ/cm^2 UVB or more (Storey et al., 2007). Viability of normal human dermal cells was

also found to be reduced after exposure to different UVR doses (Xu et al., 2010). D'Errico (2005) reported that viability of both primary fibroblasts and keratinocytes was decreased post 500 and 1000mJ/cm^2. The viability of normal human dermal fibroblasts was significantly decreased to 95%, 83% and 77% when cells were exposed to 10, 30 and 60 mJ/cm^2, respectively; viability was assessed 24h post UVR (Izykowska et al., 2009). Finally, human primary fibroblast viability was decreased to 80% after 30min of UVR and 30% after 60min of UVR (Silvana Gaiba et al., 2012).

Epidermal and dermal cells are target to UVA. Depending on their location in the body, function and metabolic characteristics keratinocytes and fibroblast may respond differently to UVR (Tyrrell and Keyse, 1990). Our results clearly show that viability of both HaCaT and 46BR.1N cells were decreased by increased UVR. A comparison was made between HaCaT and 46BR.1N cells and revealed that there was no difference when cells were exposed to low doses, e.g.15 and 30mJ/cm^2. However, 46BR.1N were more resistant to UVR when exposed to doses greater than 70mJ/cm^2. HaCaT cells were less resistant and their viability was reduced to a greater extent at high UVR doses. The differential sensitivity to UVB of keratinocytes and fibroblasts may be a result of differences in the level or repair of DNA damage in the two cell types (D'Errico et al., 2003).

In order to investigate the effect of EPA, DHA and OA on the viability of HaCaT and 46BR.1N cells, cells were treated with 10 and 50 μM of each fatty acid and viability was assessed by MTT. OA was chosen as a control fatty acid in this study because it is not an essential monounsaturated FA abundant in cell membranes (Grammatikos et al., 1994). It does not metabolise to n-3 or n-6

PUFA, EPA and DHA. .The data in this study showed that cell viability was not significantly decreased when cells were treated with OA and EPA. However, a significant decrease in cell viability was observed with an increased DHA dose. Many studies have examined the effect of fatty acids on cell viability. A decrease in the viability of pro-monocytic U937 cells treated with DHA for 24h was found to be dose-dependent (5-20 µmol/L), as reported by Yano et al. (2000). Ramos cell (Human Burkitt's lymphoma cell line) viability was decreased when it was treated with EPA (Finstad et al., 2000). The effect of AA, EPA, DHA, OA and stearic acid (SA) on viability of 14 different leukaemia cell lines was studied by Finstad et al. (1998) and they found that cell viability decreased in 10 of these cell lines when treated with AA, EPA and DHA at 30, 60 and/or 120 µM and for 3 days. However, there was no effect when the cells were treated with OA and SA. A variation in the toxic effect of fatty acids on murine and human melanoma cell viability, when treated with AA, LA, palmitic, and palmitoleic acids (Andrade et al., 2005) was also observed. Several studies on fatty acids have reported that µM of n-3PUFA concentrations are toxic to cancer cells both *in vitro* and *in vivo*. EPA at 40µM was cytotoxic to the human leukemic cells K-562 and HL-60 (Chiu and Wan, 1999).

However, other studies have claimed that EPA and DHA do not affect cell viability. DHA was not toxic to the human melanoma cell line SK-Mel 23 (Andrade et al., 2005).There was no significant effect on keratinocyte and fibroblast viability when treated with EPA and DHA 50µM for 4.5 days (Storey et al., 2005). Moreover, no significant difference in HaCaT cell viability was observed after supplementation of CLA, CLA isomers and OA (50 µM) for 4 or 5 days (Storey et al., 2007).

To assess the potential protective role of n-3 PUFA on skin cell against UVR, HaCaT and 46BR.1N were treated with OA, EPA and DHA at 10 and 50μM and viability was measured with and without 15 and 50 mJ/cm^2 24h post UVR. The results show that viability of both cell types was significantly decreased when they were treated with the fatty acid. Moreover, 50 μM of EPA and DHA produced a significant decrease in HaCaT viability following exposure to 15 and 50mJ/cm^2 UVR. Only a high concentration of DHA (50μM) produced a significant decrease in 46BR.1N viability following exposure to15 and 50mJ/cm^2 compared to the irradiated control cells (FA(-)/UVR(+))

Apoptosis is one of the pathways of cell death (Kerr et al., 1972). Henseleit et al (1996) reported that UVB can change the morphology of DNA fragmentation. APOPercentage™ kit was used to assess the DNA damage. The results in the current study show that, UVR, EPA and DHA were able to induce apoptosis in HaCaT and 46BR.1N cells. Apoptosis was significantly increased when higher doses of UVR or concentrations of PUFA were used. Takasawa et al (2005) reported that 80% of HaCaT cells underwent apoptosis post 500J/m^2 UVB or 100UVA J/m^2 at 24h (Takasawa et al., 2005). Reagan-Shaw reported that HaCaT viability was significantly decreased by UVB and apoptosis was significantly increased to 50% of un-treated cells at 24h after being treated with 15mJ/cm^2 and 50 mJ/cm^2 increased apoptosis to 66% (Reagan-Shaw et al., 2006). This was also confirmed in other studies including a time dependent experiment where apoptosis increased from 50.5% at 2h to 70% at 4h and 93% at 8h post 50mJ/cm^2 UVR (Fischer et al., 2006). Furthermore, a dose study showed an increase from 0.21% to 71.18% at 0mJ/cm^2 and 90mJ/cm^2 UVB, respectively (Reagan-Shaw et al., 2006). The apoptotic level was not significant

in primary human keratinocytes when exposed to physiological doses of UVB between 10 and 40mJ/cm^2 (Sesto et al., 2002). Serini et al (2011) reported that HaCaT apoptosis increased 3.8 fold post 60mJ/cm^2 compared to the untreated control, while, nr-HaCaT treated with DHA 30 and 50μM for 24h showed increased cell apoptosis. A further increase was observed when nr-HaCaT cells treated with DHA (50μM) were exposed to 60mJ/cm^2 UVR. EPA reduced the nr-HaCaT apoptosis in a dose-dependent manner and it did not change after exposure to UVR. A similar effect was observed with OA and LA (Serini et al., 2011). The number of human primary fibroblasts apoptosis cells was significantly increased to 21% post 30min and to 50% post 60min following exposure to UVR (Silvana Gaiba et al., 2012).

The results suggest that both UVR and the n-3 PUFA EPA and DHA decrease cell viability and increase apoptosis in a dose-dependent manner in HaCaT and 46BR.1N cells. Moreover, further decreases in viability and increased apoptosis were observed when cells were treated with n-3 PUFA and exposed to UVR.

Chapter 5. Effect of n-3 PUFA on the fatty acid composition of HaCaT keratinocytes and 46BR.1N fibroblasts

5.1. Introduction

The skin is an organ that plays important roles in protection, biosynthesis, storage, excretion, absorption and heat regulation of the body (Madison, 2003). The skin is composed of many layers and has several types of cells. Understanding how these cells are acting and regulate signalling pathways has great potential for the treatment of skin disorders such as inflammation, skin cancer and genetic abnormalities (Deyrieux and Wilson, 2007). *In vitro* models are needed to study this. These models should mimic the real physiological pathways in the skin. HaCaT keratinocytes and 64BR.1N fibroblasts were used in this project in order to study the response of skin cells under UVR and fatty acid treatments.

Primary keratinocytes can be used as an *in vitro* model; unfortunately, they provide only a short time in which to investigate and they rapidly die. Moreover, these cells require specific supplementation with growth factors in order to survive. In contrast, the immortalised human HaCaT keratinocytes can grow in normal cell culture media without any specific supplementation. HaCaT can survive in cultures for a long time and are considered immortal cells (more than 140 passages) (Boukamp et al., 1988). After multiple passages HaCaT cells are still able to retain their capacity for differentiation similar to normal keratinocytes (Boukamp et al., 1988). This offers a stable model for the study of keratinocytes (Boukamp et al., 1988). HaCaT cells have been used to study the differentiation of keratinocytes (Deyrieux and Wilson, 2007). HaCaT has also been used as a model to study the effect of UVR and assess the protective effect of inositol hexaphosphate (IP6) (Williams et al., 2011) , silibinin (Dhanalakshmi et al., 2004) and ketoprofen (Liu et al., 2007) on skin epidermal

cells against UVB and skin cancer. The composition and synthesis of lipids in normal human adult keratinocytes and HaCaT cells were studied, and the results suggest that, there was no difference between the cells in the total lipid content and distribution of major lipid classes (Schurer et al., 1993). No significant differences were observed between the lipid profile of normal keratinocytes grown in normal or low Ca^{2+} concentration in a submerged culture (Ponec et al., 1988).

The human fibroblastic cell line 46BR.1N was derived from 46BR. The parent cell line was derived from a young female with immunodeficiency (hypogammaglobulinaemia) (Teo et al., 1983). The cells were transformed with the plasmid pSV3neo expressing SV40 T-antigen and is now immortal. They are hypersensitive to DNA-damaging agents (Somia et al., 1993). 46BR.1N has been used to represent human dermal fibroblasts. 46BR.1N was used to study the effects of enoxaparin and onion extract on cytokine production in skin fibroblasts (Michał Pikuła et al., 2009). Both cells, HaCaT keratinocyte and 46BR.1N, are used in this study because they are human skin cells, immortal as opposed to normal cells, and they are not cancer cells.

Skin epidermis is an active site of lipid synthesis. The intercellular lipids of human stratum corneum are unique in composition and quite different from the lipids found in most biological membranes (Lampe et al., 1983). The three major lipids in the human stratum corneum are free fatty acids, cholesterol and ceramides (Grubauer et al., 1987). Fatty acids can be synthesised by keratinocytes *de novo*; in addition, they need to be taken up from circulation. It has been reported that an increase in fatty acid synthesis on the epidermal layer was marked during the disruption of the permeability barrier (Ottey et al.,

1995). Moreover, barrier disruption increases the activity and mRNA levels of both acetyl CoA carboxylase and fatty acid synthase, which are the key enzymes required for *de novo* fatty acid synthesis (Harris et al., 1997).

In this study, HaCaT and 46BR.1N cell lines were used to represent the epidermis and dermis layers, respectively. They were cultured and treated with n-3 PUFA to achieve and answering the following:-

1- Is there any difference between HaCaT and 46BR.1N with regard to their fatty acid composition?
2- Do both HaCaT and 46BR.1N up take the n-3 PUFA treatment?
3- How does the n-3 PUFA treatment affect the fatty acid profile of both cell lines?

5.2. Materials and methods

All information relevant to the experiments reported in this study is presented in section 2.2. Cell culture and passaging in section 2.2.3.2 and cell counting in section 2.2.3.2. Section 2.2.3.4 describes the treatment of cells with fatty acids and section 2.2.3.5 shows the collection of cells and media. Fatty acid analysis is reported in section 2.4. Preparation of sample was described in section 2.3.3.1, lipid extraction in section 2.3.3.2 and section 2.3.3.5 shows the preparation of fatty acid methyl esters for GC analysis.

5.3. Results

5.3.1. Fatty acid profile of HaCaT and 46BR.1N cells

The fatty acid profiles of HaCaT and 46BR.1N cells are shown in Table 5.1. Thirty one fatty acids were detected by GC analysis: The SFA group comprised 37.11 ± 9.16% of total fatty acids in HaCaT keratinocytes but it was

Table 5.1. Fatty acid profile of HaCaT keratinocytes and 46BR.1N fibroblasts. Results are expressed as % weight of total fatty acid (mean ± SD) of n=3 independent experiments

FATTY ACID	HaCaT keratinocytes	46BR.1N fibroblasts
C12:0	0.05 ± 0.04	0.16 ± 0.11
C14:0	1.52 ± 0.32	1.50 ± 0.39
C15:0	0.11 ± 0.07	0.41 ± 0.05
C16:0	18.96 ± 2.48	20.68 ± 2.36
C17:0	0.26 ± 0.12	1.17 ± 0.13
C18:0	15.32 ± 8.09	26.83 ± 5.30
C20:0	0.23 ± 0.08	0.42 ± 0.05
C23:0	0.03 ± 0.01	0.05 ± 0.02
C24:0	0.64 ± 0.53	0.72 ± 0.26
ΣSFA	**37.11 ± 9.16**	**51.94 ± 8.68**
C14:1	0.03 ± 0.10	0.04 ± 0.03
C16:1	9.85 ± 4.90	1.79 ± 0.33
C17:1	0.53 ± 0.08	0.46 ± 0.31
C18:1n-9t	0.09 ± 0.03	0.23 ± 0.10
C18:1n-9c	26.47 ± 1.77	18.23 ± 2.98
C18:1n-7	14.85 ± 5.17	5.24 ±0.73
C20:1n-9	1.51 ± 0.39	0.34 ± 0.08
C22:1n-9	0.48 ± 0.32	0.35 ± 0.03
C24:1	0.35 ± 0.24	0.39 ± 0.21
ΣMUFA	**54.22 ± 8.82**	**27.07 ± 4.79**
C18:2n-6t	0.10 ± 0.09	0.23 ± 0.04
C18:2n-6c	2.19 ± 1.00	4.25 ±1.10
C18:3n-6	0.13 ± 0.25	0.08 ± 0.08
C20:3n-6	0.43 ± 0.15	0.65 ± 0.08
C20:4n-6	3.04 ± 1.69	9.40 ± 3.61
ΣN-6PUFA	**5.88 ± 2.64**	**14.62 ± 5.10**
C18:3n-3	0.03 ± 0.01	0.19 ± 0.04
C20:3n-3	0.33 ± 0.08	0.49 ± 0.10
C20:5n-3	0.25 ± 0.09	0.94 ± 0.34
C22:5n-3	1.0±0.26	1.82 ± 0.45
C22:6n-3	1.13 ± 0.23	2.68 ± 0.47
ΣN-3PUFA	**2.72 ± 0.49**	**6.11 ± 1.40**
C20:2	0.18 ± 0.09	0.19 ± 0.04
C22:2	0.04 ± 0.02	0.04 ± 0.01

higher in 46BR.1N fibroblasts at 51.94 ± 8.68%. A total of 9 SFAs were detected : C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C23:0 and C24:0, with the main ones being C16:0 and C18:0. The amounts of these fatty acids in 46BR.1N were more than in HaCaT (Table 5.1)

MUFA were 54.22 ± 8.82% of total fatty acids in HaCaT cell and less were found (27.07±4.79%) in 46BR.1N. Nine fatty acids were detected in this group; C14:1, C16:1, C17:1, C18:1n-9t, C18:1n-9c, C18:1n-7, C20:1n-9, C22:1n-9 and C24:1. The main fatty acid was C18:1n.9c (26.47 ± 1.77%and 18.23 ± 2.98%) followed by C18:1n-7 (14.85% ± 5.17 and 5.24±0.73%) on HaCaT and 46BR.1N, respectively.

N-6 PUFAs were 5.88 ± 2.64% and 14.62 ± 5.10% of total HaCaT and 46BR.1N fatty acids, respectively. This group comprised five PUFA; C18:2n.6t, C18:2n.6c, C18:3n-6, C20:3n-6 and C20:4n-6. C20:4n-6 was the main n-6 PUFA in both HaCaT and 46BR.1N cells with 3.04 ± 1.69% and 9.40 ± 3.61%, respectively. Followed by C18:2n-6c at 2.19 ± 1.0% in HaCaT cell and at 4.8 ± 1.1% on 46BR.1N.

Finally, 5 n-3 PUFAs were found in both cell lines. These were C18:3n-3, C20:3n-3, C20:5n-3, C22:5n-3 and C22:6n-3. In HaCaT n-3 PUFA were 2.72 ± 0.49% of total while in 46BR.1N the total % was higher (6.11 ± 1.40%). In HaCaT C22:6n-3 was found 1.13 ± 0.23% followed by C22:5n-3 at 1.0 ± 0.26%. The same fatty acids were detected in 46BR.1N and C22:6n-3 was 2.68 ± 0.47% followed by C22:5n-3 at 1.82± 0.45% and C20:5n-3 at 0.94± 0.34%.

5.3.2. Effect of n-3 PUFA treatment on HaCaT fatty acid profile

These experiments were performed in order to assess the effect of EPA and DHA on the fatty acid profile of the HaCaT cell line. The cells were cultured in Petri dishes and treated with n-3 PUFA (10 and 50μM) for 72h. Oleic acid was chosen as a control treatment for the experiments. After that the cells were collected and lipid extracts were analyzed by GC for fatty acid methyl ester.

There was no statistically significant difference on the levels of SFA, MUFA and n-6 PUFA after 72h of treatment with OA, EPA and DHA (10 or 50 μM) compared to non-treated control cells. Detailed data are shown in Appendices 3.1, 3.2 and 3.3. However, the UVR and fatty acid treatments affected the n-3 PUFA specificities;

The OA treatment did not affect the level of n-3 PUFA group comparing to control cells. These results are shown in Figure 5.1.

Statistical analysis showed no a significant increase on total n-3 PUFA when HaCaT treated with 10μM EPA. However, further increase was shown when the cells were treated with EPA 50μM (11.02 ± 1.98 %, $p \leq 0.001$) these results are shown in Figure 5.1.

When the HaCaT cells were treated with DHA 10μM there was no statistically significant increase on the total n-3 PUFA level. However, the amount of n-3 PUFA group was significant increased from 2.72±0.49% in untreated HaCaT cells to 10.63 ± 4.96% ($P \leq 0.01$) follows treatment with 50μM DHA. The results are shown in Figure 5.1.

When we looked of individual n-3 PUFA we found that the EPA and DHA treatment had affected the levels of EPA, DPA and DHA specifically:

There was no effect on C20:5 n-3 when HaCaT cells were treated with OA 10 and 50μM (Figure 5.2.). When HaCaT were treated with EPA 10μM there was no significant increase on C20:5 n-3 concentration. Significantly increased on C20:5 n-3 ($p \leq 0.001$) was observed when cells were treated with EPA 50μM compared to non-treated cells (Figure 5.2). Similar, no significantly increase on C20:5n-3 was observed when HaCaT cells were treated with 10μM DHA. But significant increases on C20:5 n-3 ($p \leq 0.05$) were observed when the cells were treated with 50μM DHA (Figure 5.2).

The levels of C22:5 n-3 were not significantly increased when HaCaT cells were treated with OA or DHA (10 and 50μM) (Figure 5.3). They were significant increased when the cells were treated with EPA 10μM ($p \leq 0.01$) and 50μM ($p \leq 0.001$). Results are shown in Figure 5.3

Finally, statistical analysis showed that there was no effect of OA and EPA (10 and 50 μM) on the level of C22:6 n-3 as shown in Figure 5.4. DHA treated HaCaT cells showed increased levels of C22:6 n-3. This was not statistically significant when cells were treated with 10μM DHA but when treated with DHA 50μM this became significant ($P \leq 0.01$). Results are shown in Figure 5.4.

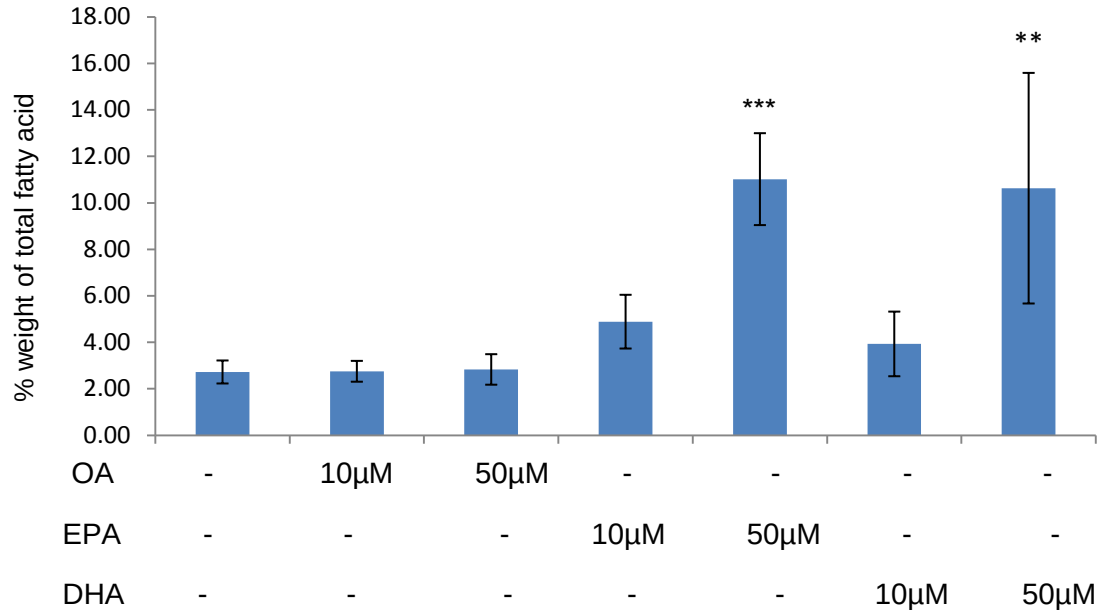


Figure.5.1. Effect of fatty acid treatment, oleic acid (OA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the level of total n-3 PUFA in HaCaT cells. Results are shown as mean ± SD of 3 independent experiments, each analysed in duplicate. **=p≤0.01, ***=p≤0.001, comparing data to non-treated group.

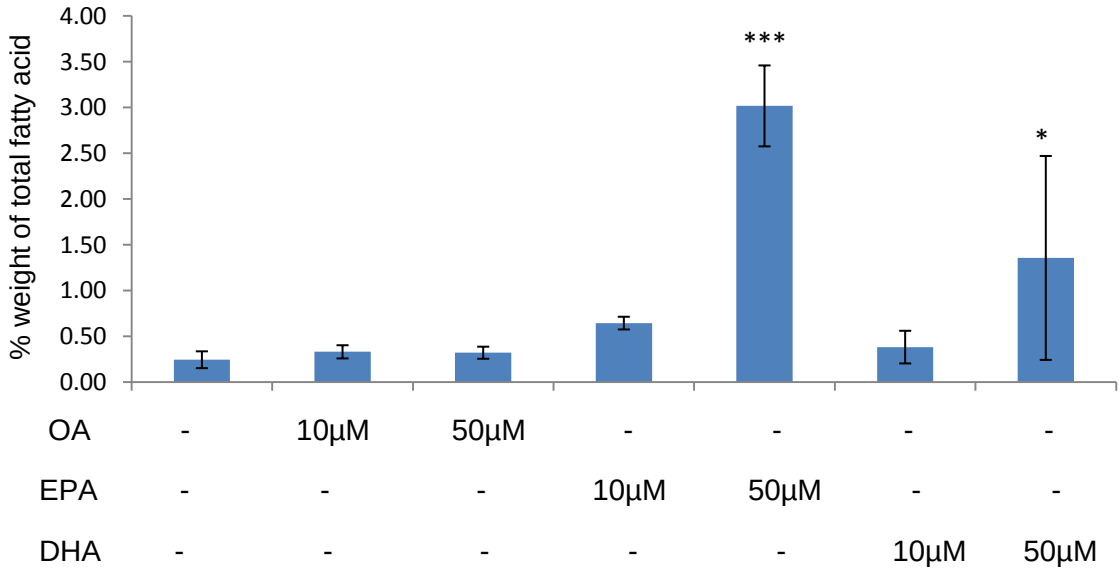


Figure 5.2. Effect of fatty acid treatment, oleic acid (OA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the level of eicosapentaenoic acid (EPA,C20:5 n-3) on HaCaT cells. Results are shown as mean ± SD of 3 independent experiments, each analysed in duplicate. *=p≤0.05, ***=p≤0.001, comparing data to non-treated group.

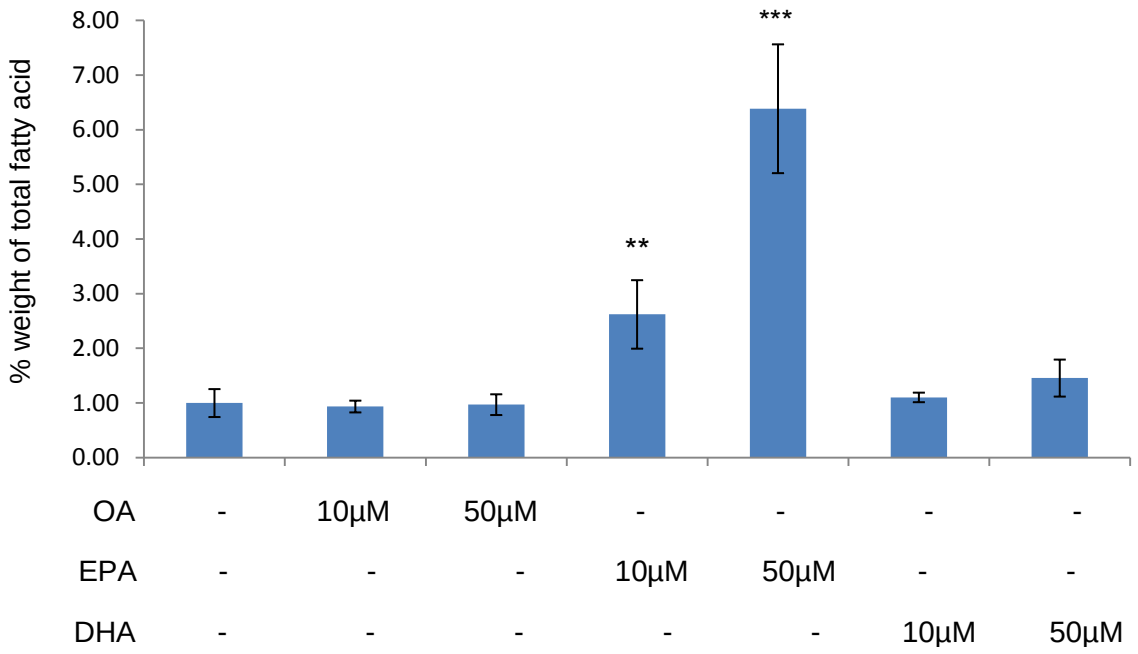


Figure.5.3. Effect of fatty acid treatment, oleic acid (OA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on docosapentaenoic acid (DPA, C22:5 n-3) in HaCaT cells. Results are shown as mean ± SD of 3 independent experiments, each analysed in duplicate. **=p≤0.01, ***=p≤0.001, comparing data to non-treated group.

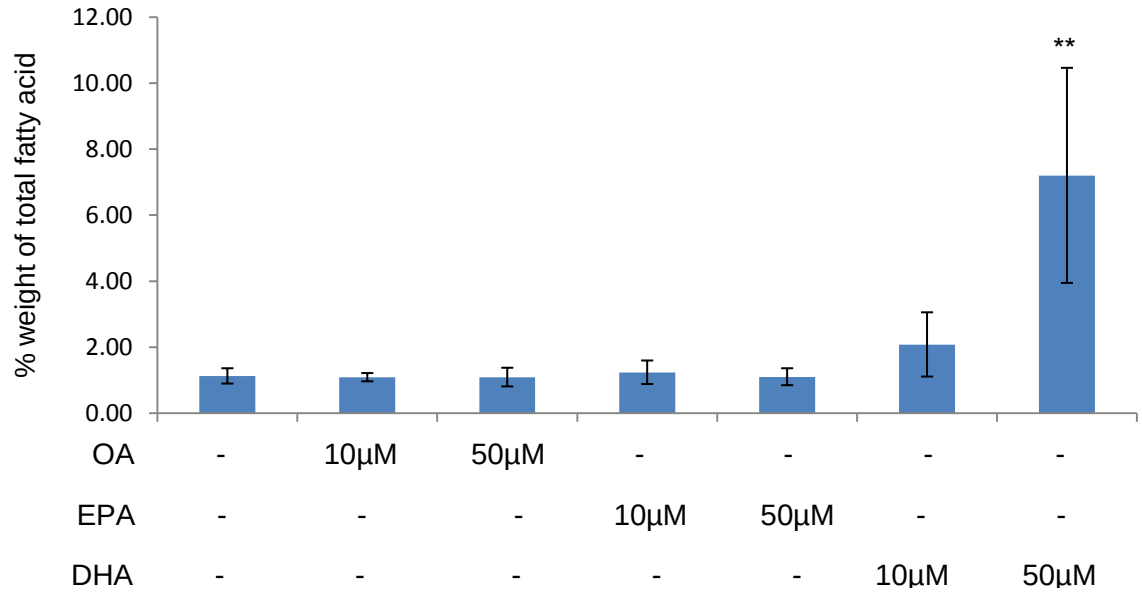


Figure.5.4. Effect of fatty acid treatment, oleic acid (OA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the level of docosahexaenoic acid (DHA, C22:6 n-3) in HaCaT cells. Results are shown as mean ± SD of 3 independent experiments, each analysed in duplicate. **=p≤0.01, comparing data to non-treated group.

5.3.3. Effect of n-3 PUFA treatment on 46BR.1N fatty acid profile

These experiments were performed in order to assess the effect of fatty acid treatment (OA, EPA and DHA) on the fatty acid profile of the 46BR.1N cell line. The cells were cultured in Petri dishes and treated with the fatty acids (10 and 50μM) for 72h. Statistical analysis shown that there were no effects on the saturated, monounsaturated or n-6 PUFA levels in 46BR.1N cells, but there were changes in some n-3 PUFA. Specifically:

There were no statistically significant differences in the n-3 PUFA group after 72h treatment with OA (10 and 50 μM) compared to non-treated control (Figure 5.5). Also no statistically significant increases in the levels of n-3 PUFA post treatment with a low dose of EPA and DHA (10μM). Significant increases on n-3 PUFA were observed when cells were treated with EPA 50μM ($p \leq 0.01$) and DHA 50μM ($p \leq 0.01$) compared to non-treated cells (Figure 5.5). Detailed data are shown in Appendixes 3.4, 3.5 and 3.6.

Furthermore, the fatty acid treatment changed the levels of some fatty acids in 46BR.1N specificities:

The concentration of C18:1n-9c was significantly increased to 31.74 ± 3.09% when the cells were treated with OA 50μM compared to non-treated cell ($P \leq 0.05$); but there was no significant different when treated with EPA and DHA as shown in Figure 5.6. The amount of C20:3n-6 was significantly increased when the cells were treated with DHA 10μM ($p \leq 0.05$) and 50 μM ($p \leq 0.05$) and compared to control groups. Results are shown in Figure (5.7). The concentration of C18:3n-3 was not affected by OA and DHA. However, EPA 50 μM increased it significantly ($p \leq 0.05$) (Figure 5.8).

Figure 5.9 shows the effect of fatty acid treatment on C20:5n-3 levels in 46BR.1N cells. The level of C20:5n-3 did not change with OA and DHA treatment. However, there was a significant increase when the cells were treated with EPA 10μM ($p \leq 0.001$) and 50μM ($P \leq 0.001$). Also, a significant increase was observed in the amount of C22:5n-3 after treating the cells with EPA 10μM ($p \leq 0.05$) and 50μM ($P \leq 0.001$) and compared to control group. (Figure 5.10)

Finally, an increase in the concentration of C22:6n-3 was observed in 46BR.1N cells treated with DHA (50μM). This was significantly increased compared to the control group ($P \leq 0.001$) (Figure 5.11)

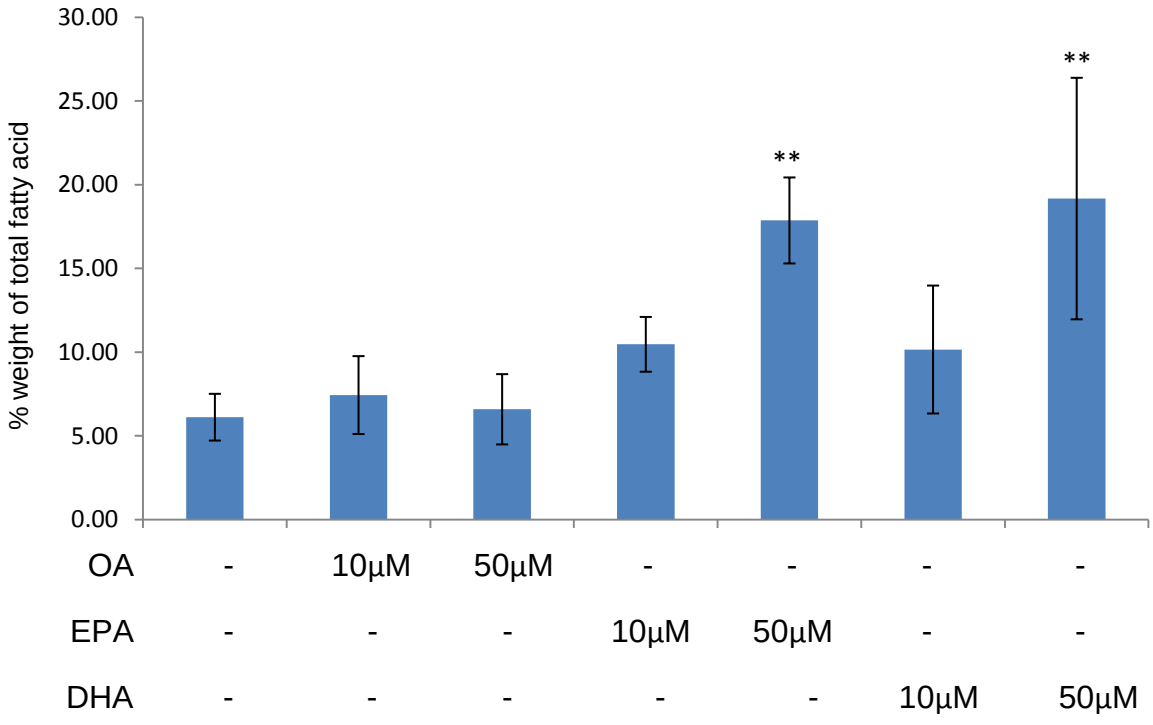


Figure.5.5. Effect of fatty acid treatment, oleic acid (OA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the level of total n-3 PUFA in 46MR.1N fibroblasts cells. Results are shown as mean ± SD of 3 independent experiments, each analysed in duplicate. **=p≤0.01, comparing data to non-treated group.

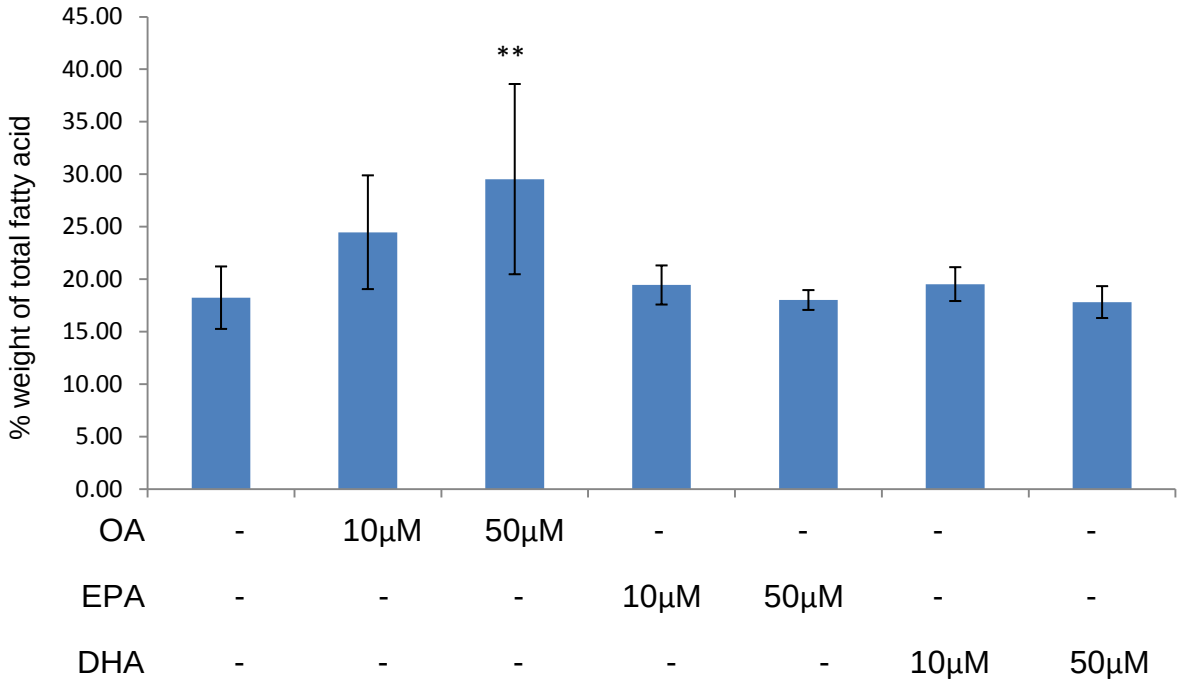


Figure 5.6. Effect of fatty acid treatment, oleic acid (OA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the level of oleic acid (OA, C18:1n-9c) in 46BR.1N fibroblast cells. Results are shown as mean ± SD of 3 independent experiments, each analysed in duplicate. **=p≤0.01, comparing data to non-treated group

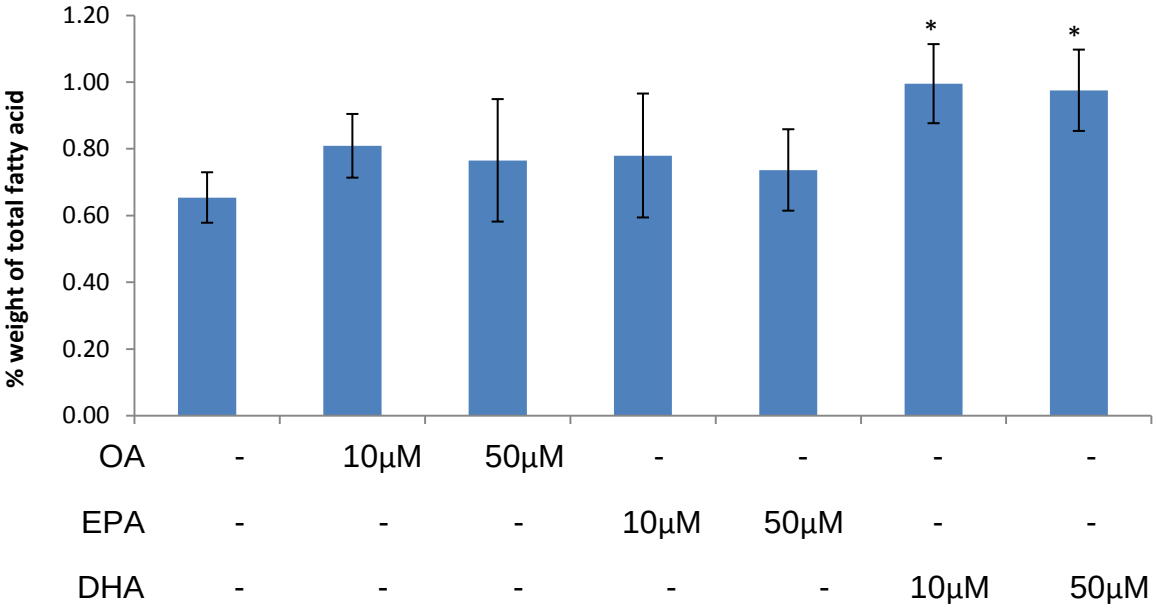


Figure 5.7. Effect of fatty acid treatment, oleic acid (OA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the level of Dihomo-γ-linolenic (DG LA,C20:3 n-6) on 46BR.1N fibroblast cells. Results are shown as mean ± SD of 3 independent experiments, each analysed in duplicate. *=p≤0.05, comparing data to non-treated group.

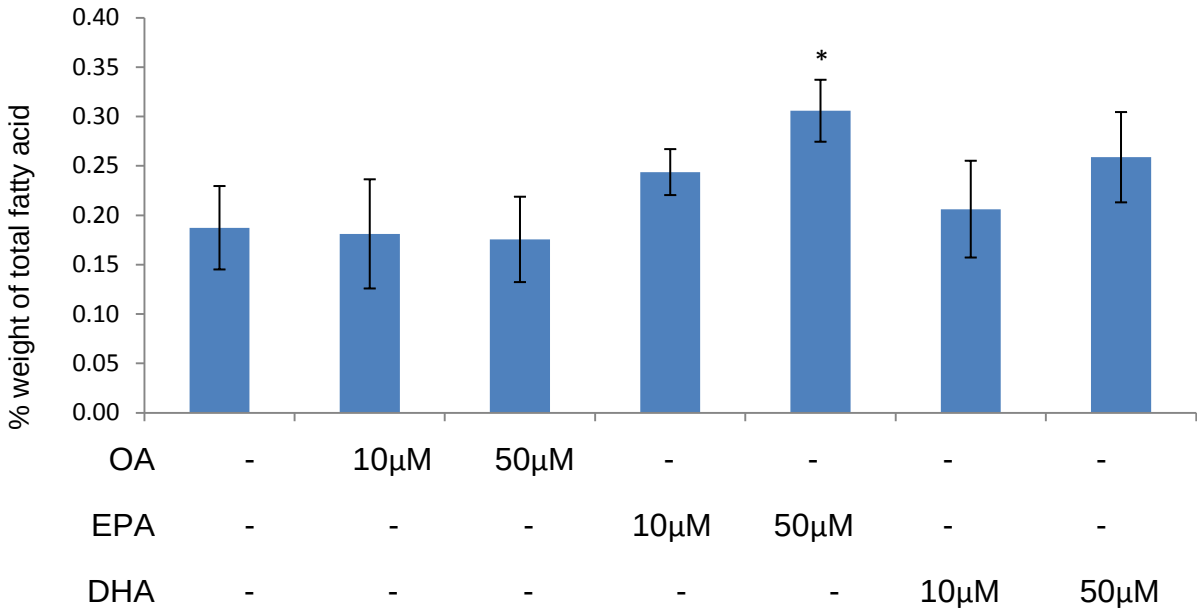


Figure.5.8. Effect of fatty acid treatment, oleic acid (OA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the level of α-linolenic acid (ALA, C18:3 n-3) on 46BR.1N fibroblast cells. Results are shown as mean ± SD of 3 independent experiments, each analysed in duplicate. *=p≤0.05, comparing data to non-treated group.

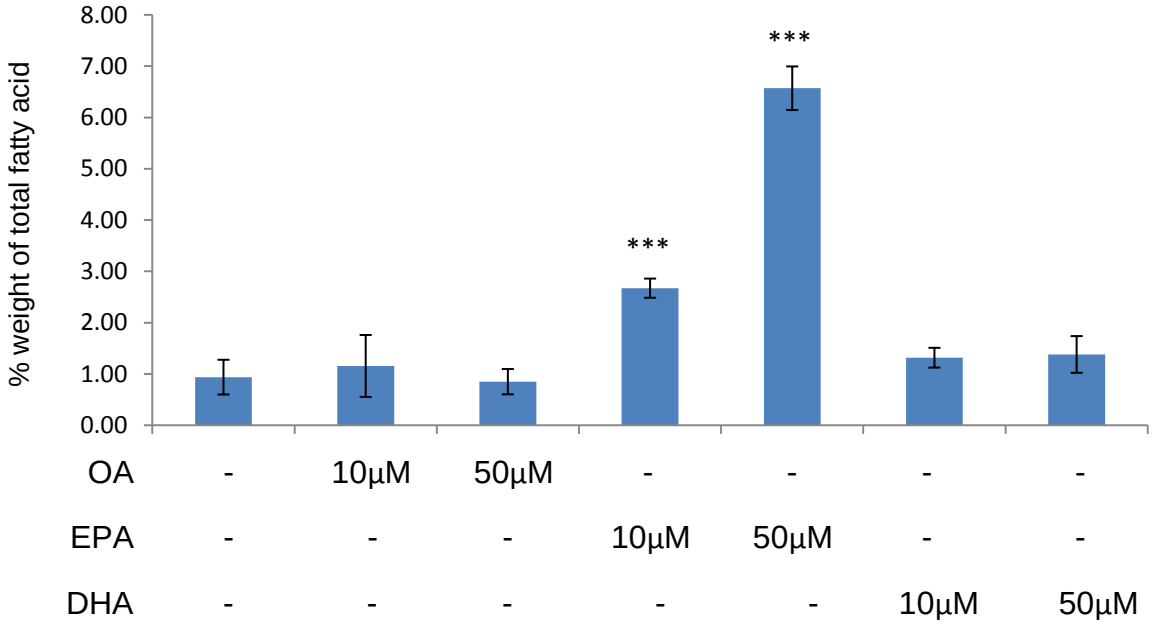


Figure 5.9. Effect of fatty acid treatment, oleic acid (OA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the level of eicosapentaenoic acid (EPA, C20:5 n-3) on 46BR.1N fibroblast cells. Results are shown as mean ± SD for 3 independent experiments, each analysed in duplicate. ***=p≤0.001, comparing data to non-treated group.

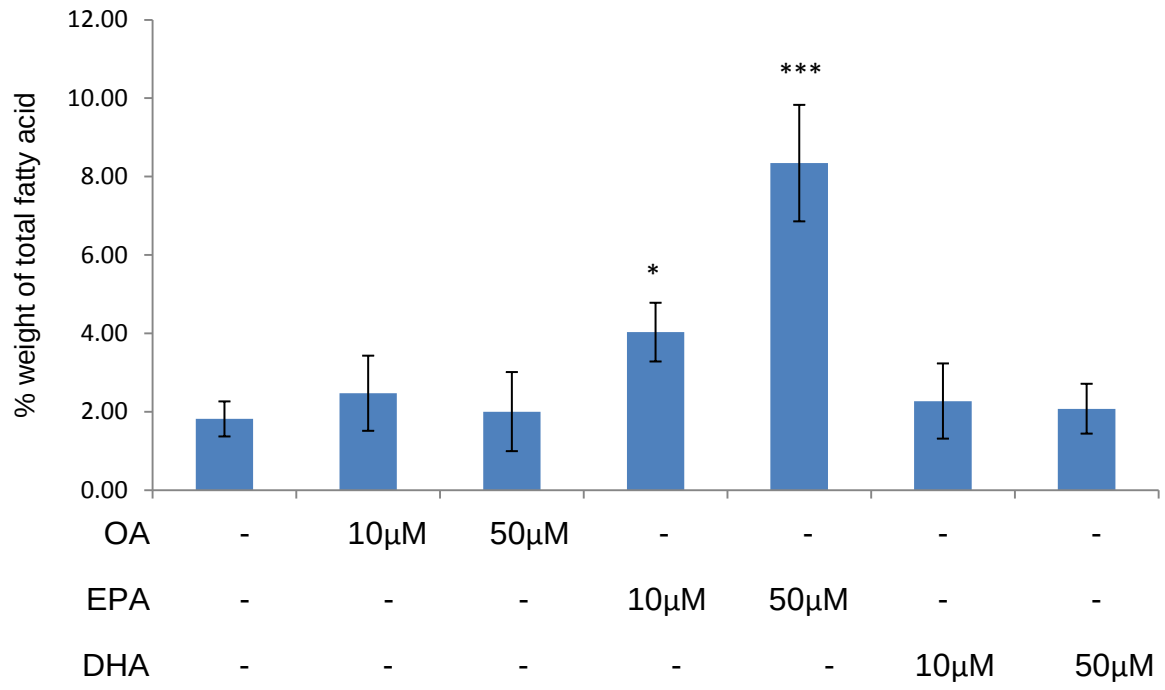


Figure 5.10. Effect of fatty acid treatment, oleic acid (OA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)on the level of docosapentaenoic acid (DPA, C22:5 n-3) in 46BR.1N fibroblast cells. Results are shown as mean ± SD of 3 independent experiments, each analysed in duplicate. *=p≤0.05, ***=p≤0.001, comparing data to non-treated group.

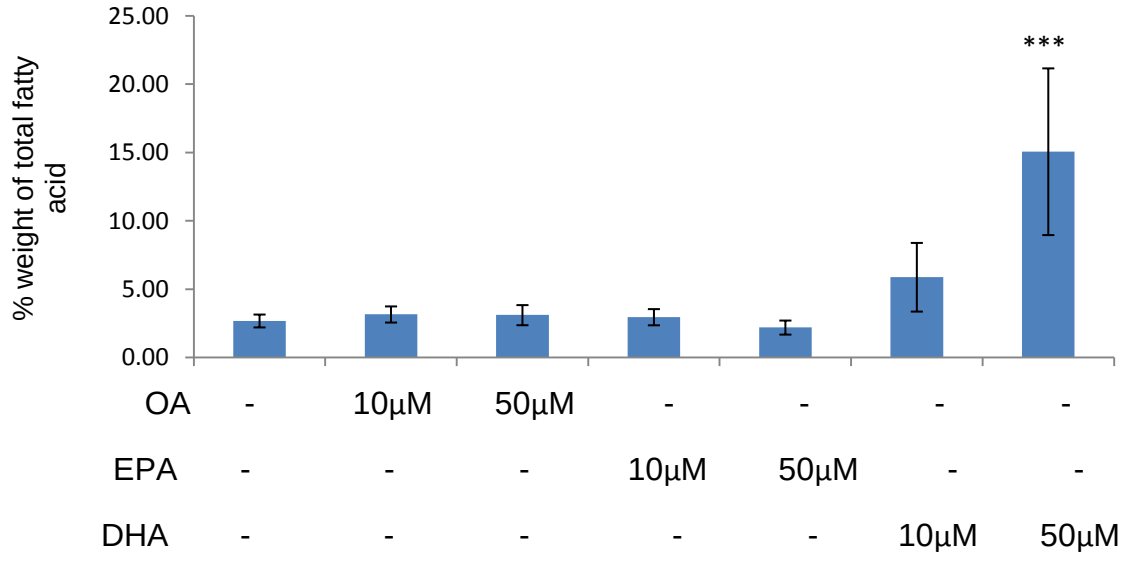


Figure 5.11. Effect of fatty acid treatment, oleic acid (OA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the level of docosahexaenoic acid (DHA, C22:6 n-3) in 46BR.1N fibroblast cells. Results are shown as mean ± SD of 3 independent experiments, each analysed in duplicate. ***=p≤0.001, comparing data to non-treated group.

5.4. Discussion

In order to assess the effect of EPA and DHA on the fatty acid profile of HaCaT cells, the baseline concentration was analysed. The results show that C16:0 (19%) and C18:0 (15.3%) were the main SFA. Terashi, et al (2000) reported that in primary human epidermal keratinocytes from the face grown in culture C16:0 (17.5%) was the main SFA followed by C18:0 (16%) (Terashi et al., 2000). Also, in human keratinocytes C16:0 (16.7%) was the predominant SFA, followed by C18:0 (9.3%), when cells were cultured in normal conditions (Ponec et al., 1988). The amount of C16:0 (23.1%) in cultured oral keratinocytes and normal oral mucosal keratinocytes (27.18%) was higher in that found in our study (Moriyama et al., 2011). C18:1n-9t (26.47%) was found to be the main MUFA. This level was higher than that found in C18:1n-9 (16.0%) in cultured oral keratinocytes and lower than that found in human keratinocyte (Ponec et al., 1988). In this study, the levels of C18:2 n-6 (2.2%) and C20:4n-6 (3.0%) were lower than in cultured oral keratinocytes which were 27.4% and 6.3% respectively. Finally, the level of EPA and DHA was about 0.25% and 1.13% respectively in the HaCaT cells used in the present study and this was similar to the level found in cultured oral keratinocytes. Furthermore, it has been reported that the amount of EPA and DPA was 0% in HaCaT keratinocytes (Storey et al., 2005).

The fatty acid profile of 46BR.1N dermal skin fibroblasts was analysed by GC. Thirty one fatty acids were detected, and C18:0 (26.8%) was the main SFA, followed by C16:0 (20.7%). A similar result was found in cultured human skin fibroblasts and the main SFA was C18:0 (22.4%), followed by C16:0 (19.9%) (Williard et al., 2001). In another study on human skin fibroblasts, C18:0 (29.8%)

was the main SFA, followed by C16:0 (27.9%) (Yu-Poth et al., 2005). Our results show that C18:1n-9 was the main MUFA at 18.2% of total fatty acids. This was lower than the level of C18:1n-9 (22.4% and 24.7%) found in cultured human skin fibroblasts (Williard et al., 2001, Yu-Poth et al., 2005) and C18:1n-9 (25.52%) in Indian muntjac fibroblasts (Higgins et al., 1999). Among the 10 PUFAs detected in 46BR.1N, AA (9.4%), LA (4.5%), EPA (0.94%), DPA (1.82%) and DHA (2.68%) were the main ones. Compared to what was found in cultured human skin fibroblasts, AA (11.6%) and DHA (4.2%) were higher, and LA (2.6%), EPA (0.3%) and DPA (1.4%) were lower than in 46BR.1N (Williard et al., 2001). Also, Indian muntjac fibroblasts showed a higher amount of DHA (3.2%) and a lower amount of EPA (0.3%) compared to 46BR.1N (Higgins et al., 1999).

The results of this study show that there were no significant changes in the concentration of fatty acids when HaCaT cells were treated with 10 and 50 μM OA. Although there were trends showing increased levels of OA following this treatment, this failed to show any statistical significance. Furthermore, the result shows that the fatty acid composition in HaCaT was not affected when treated with OA, which was chosen as a control (Appendix 3.1). In contrast, HaCaT cells treated with EPA and DHA show significant increases in these fatty acids (Figures 5.2 and 5.4). The same results were found when HaCaT keratinocytes were treated with 50 μM OA, EPA and DHA (Storey et al., 2005).They reported that the level of EPA and DHA was increased from 0% to 3.4% and 6.3% respectively. In addition to an increase in EPA level after EPA supplementation, DPA was also increased in the EPA treated cells, while DHA treatment showed an increase in DHA and EPA but not DPA. A similar result was found by Storey et al (2005) who reported that the level of EPA was 0% in

control HaCaT cells but increased to 2.4% when cells were treated with 50 µM DHA. DPA concentration was increased from 1.5% (mol) to 25.5% when normal human keratinocytes were treated with 50 µM EPA while DHA did not increase.

The concentration of OA on 46BR.1N increased significantly after being treated with 50µM OA (Figure 5.6). This was also found in Indian muntjac fibroblasts when treated with 50µM OA. In this study, the concentration of OA increased from 25.52% to 37.99%. This was not in agreement with what was reported by Storey et al. (2005). Who found that when treating CCD922SK fibroblasts (derived from normal human breast skin) with 50 µM OA there was no significant effect on the concentration of OA. Treated cells with OA did not affect the concentration of any other fatty acid. Meanwhile, treating the cells with EPA significantly increased EPA and DPA, and treating the cells with DHA significantly increased DHA only. An increase in EPA and DHA was observed in Indian muntjac fibroblasts treated with 50 µM of each fatty acid. EPA and DHA were increased from 0.23% and 3.2% to 9.88% and 14.35%, respectively (Higgins et al., 1999). Furthermore, EPA and DHA levels were increased from 5.7% and 3.2% to 11.1% and 17.4%, respectively, when CCD922SK fibroblasts were treated with 50 µM EPA and DHA (Storey et al., 2005). It has also been reported that only trace amounts of EPA, DPA and DHA were present at basal levels in fibroblasts. However, they increased significantly, when human skin fibroblasts were treated with 0.4mmol/L of EPA or DHA (Yu-Poth et al., 2005). An increase in the concentration of DPA only was noted after EPA treatment, this could be a result of elongation of EPA or saturation of DHA. Furthermore, there were no significant increases in either EPA or DPA when cells were treated with DHA; this indicated the retro-conversion of DHA to EPA, which

takes place in peroxisomes by Δ4 enoyl reductase and Δ2, Δ3 enoyl CoA isomerise enzymes.

Overall, the aim of this study was achieved; cells were packed up the fatty acid treatment and the content of EPA and DHA was increased in the cells. The fatty acid profile in un-treated cells shows that the content of n-3 and n-6 PUFA was higher in 46BR.1N compared with HaCaT keratinocytes, specifically the levels of LA, AA, EPA, DPA and DHA. In contrast, OA was lower in 46BR.1N, and this may explain the significant increase inOA afterbeing treated with 50 µM OA.

Chapter 6. Effect of UVR and n-3 PUFA on prostanoid production in skin cells

6.1. Introduction

Prostanoids are generated from the action of cyclooxygenase (COX-1 and COX-2) and prostanoid synthases on AA, EPA and DHA (Schumert et al., 1988). COX-2 is often implicated in inflammatory diseases that are characterised by oedema and tissue injury due to the release of many inflammatory cytokines and chemotactic factors (Tanaka et al., 1997, Arslan and Zingg, 1996). It has been suggested that prostanoids inhibit apoptosis (Sheng et al., 1998) and increase the metastatic potential of cancer cells (Kakiuchi et al., 2002).

There are many factors that can influence eicosanoid producing enzymes. UVR can induce COX-2 and increase the PG levels in the skin (Buckman et al., 1998). It has been reported that increases in COX-2 expression in human skin epidermis occurred when human subjects were irradiated with up to four times their MED and biopsied 24 h later (Rhodes et al., 2009). Furthermore, increased COX-2 protein was noted when cultured human keratinocytes were exposed to 30mJ/cm^{2}UVB (Buckman et al., 1998). It has also been reported that UVB exposure up-regulated the expression of COX-2 and increased protein levels of COX-2 on culture HaCaT cells post 30mJ/cm^2 UVB (Lee et al., 2013). UVR can affect the COX-2 expression via activation of multiple signal transduction pathways including the following: (1) extracellular molecules (such as growth factor receptors (GFR), (2) intracellular signaling pathways (such as the mitogen-activated protein kinases (MAPKs), and (3) extraceullar signal-regulated protein kinases (ERK) that ultimately result in enhanced production of effector proteins including cytokines, growth factors, and MMPs.

Keratinocyte growth factor (KGF) is a member of the fibroblast growth factor (FGF) family and appears to represent a key mediator of epithelial growth

and differentiation (Finch et al., 1989). Secreted from cells of mesenchymal origin, it elicits its activity specifically on epithelial cells through binding to the keratinocyte growth factor receptor (KGFR) (Miki et al., 1992). An activation on GFR was observed in NIH3T3 cells when they were exposed to 25-50mJ/cm^2 UVB. (Marchese et al., 2003). It has been shown that MAPK pathways lead to proto-oncogene expression, and members of the MAPK family play a role in chemical carcinogen induced COX-2 gene expression (Yang et al., 2000; Arbabi et al., 2000). It has also been reported that extraceullar signal-regulated protein kinases (ERK) and p38 were significantly increased in cultured human keratinocytes and HaCaT cells post UVR treatment (Chen et al., 2001, Mutou et al., 2010).

Peroxisome proliferator-activated receptors (α, β and γ) (PPAR) are nuclear hormone receptors and ligand-activated transcription factors that regulate target gene expression by binding to specific peroxisome proliferator-response elements (PPREs) (Berger and Moller, 2002). It has been suggested that PPARγ mediates the COX-2 expression in human synovial fibroblasts (Kalajdzic et al., 2002), and UVB increases COX-2 expression via PPARγ activation on keratinocytes (Zhang et al., 2005) and in normal primary human keratinocytes (Konger et al., 2010). On the other hand, UVB has been shown to down-regulate PPAR-α and PPAR-β (Kippenberger et al., 2001).

Furthermore, it has been reported that PUFAs and their oxidised products are able to bind and activate all three isoforms of PPARs (Wahli et al., 1995). it has also been reported that EPA and GLA are able to up-regulate COX-2 expression via PPARγ in HaCaT keratinocytes (Chene et al., 2007). Moreover,

it has been found that EPA inhibits the expression of COX-2 in many systems including human skin (Kim et al., 2006).

This part of the study aimed to explor the eicosanoid production in HaCaT keratinocytes and 46BR.1N fibroblasts and to answer the following:

A) What kind of prostanoids are produced by these cells?
B) What is the effect of UVR on the production of these prostanoids?
C) What is the effect of n-3 PUFA on the production of these prostanoids with and without UVR treatment?

6.2. Materials and methods

All information relevant to the experiments reported in this study is presented in section 2.2. Cell culture and passaging are in section 2.2.3.2 and cell counting in section 2.2.3.2. Exposure of cells to UVR described in section 2.2.3.3.2. Section 2.2.3.4 describes the treatment of cells with fatty acids and section 2.2.3.5 shows the collection of cells and media. Prostanoids analysis is reported in section 2.4 including sample preparation and LC/ESI-MS/MS analysis.

6.3. Results

6.3.1. Profile of prostanoids produced by HaCaT and 46BR.1N

Cells were cultured, treated with 10 and 50μM OA, EPA and DHA, exposed to 15mJ/cm^2 UVR and the media were analysed for prostanoids as described previously. 20 Prostanoids were screened for and 7 prostaglandins were detected: PGE_1, PGD_1, PGE_2, PGD_2, PGE_3 and PGD_3 (Table 6.1). $PGF_{2\alpha}$ also was detected in low concentration which was close to the quantitation limit of the method, so it was difficult to accurately determine its true abundance. Also, a number of prostaglandin deactivation products were detected (Table 6.1). Results in Table 6.1 show the concentration (pg/million cell) of PG and their main deactivation products. It is clear that 46BR.1N produced more prostaglandins than HaCaT cells. PGE_2 was found to be the most abundant PG in both cell lines.

Table 6.1. Profile of prostanoids produced by HaCaT and 46BR.1N. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate).

Compound	HaCaT(pg/ million cell)	HaCaT (% total PG)	46BR.1N (pg/ million cell)	46BR.1N (% total PG)
PGE_1	0.84 ± 0.41	1.2	4.4 ± 0.09	2.0
PGD_1	0.63 ± 0.43	0.9	1.4 ± 0.09	0.5
PGE_2	7.96 ± 3.18	12.2	44.2 ± 23.0	17.6
PGD_2	1.48 ± 1.19	1.98	17.1 ± 9.71	4.8
PGE_3	1.39 ± 0.19	2.5	4.7 ± 1.04	2.7
PGD_3	1.18 ± 1.16	1.99	10.7 ± 7.46	7.0
15-keto PGE_2	6.28 ± 3.23	11.4	13.1 ± 7.99	11.0
13,14dihydro-15-PGE_1	1.85 ± 1.47	2.3	3.7 ± 2.46	2.9
13,14dihydro-15-PGE_2	32.3 ± 19.56	46.5	55.2 ± 37.8	42.3
13,14dihydro-15-$PGF_{2\alpha}$	14.6 ± 12.7	19.1	20.0 ± 2.04	9.1

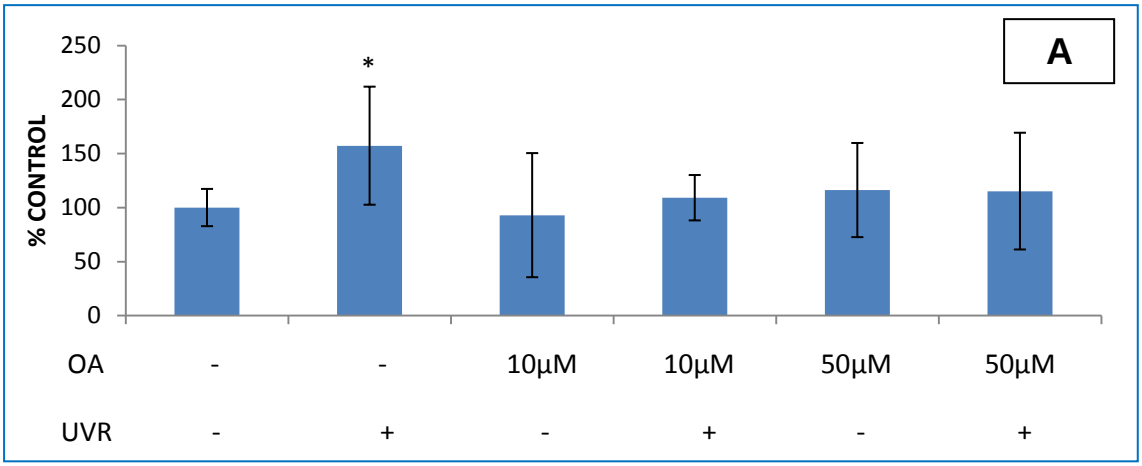
6.3.2. The effect of UVR and fatty acid treatment on prostanoids produced by HaCaT keratinocyte

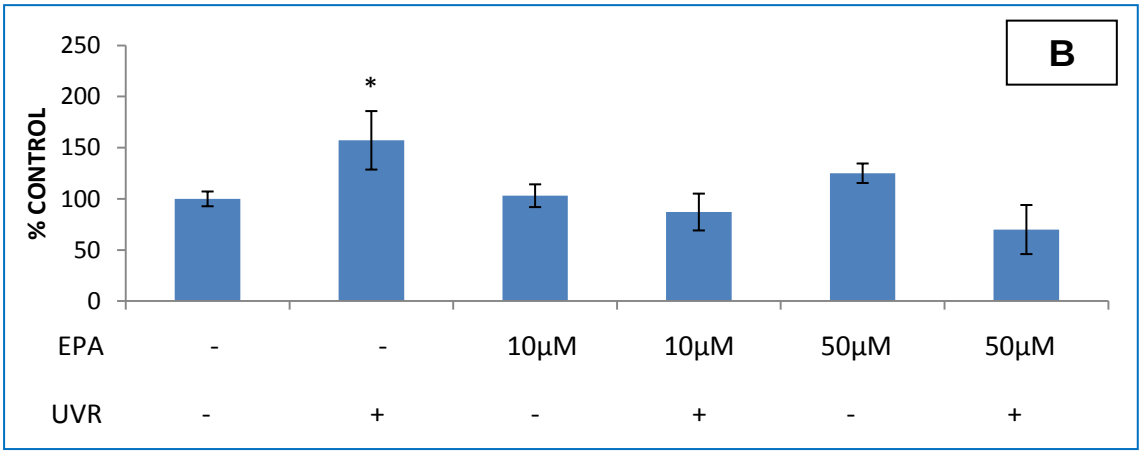
6.3.2.1. The effect of UVR and fatty acid treatment on series-1 prostanoids.

PGE_1, PGD_1 and 13,14-dihydro-15-keto PGE_1 are all products of DGLA, their levels were increased in HaCaT post UVR exposure. However only PGE_1 was statistically significantly higher ($p \leq 0.05$) compared to non-irradiated control (FA(-)/UVR(-)) (Figure 6.1-3). HaCaT cells treated with 10 and 50µM of OA showed no significant changes in PGE_1, PGD_1 and 13,14-dihydro-15-keto PGE_1. Similarly there were no significant effects of OA (10 and 50µM) on the levels of PGE_1, PGD_1 and 13,14-dihydro-15-keto PGE_1 post UVR compared to irradiated control (FA (-)/UVR(+)).Figures 6.1A, 6.2A and 6.3A

There was no significant decrease in PGE_1, PGD_1 and 13,14-dihydro-15-keto PGE_1 when HaCaT cells were treated with 10 and 50µM of EPA Figures (6.1B, 6.2B and 6.3B) . But PGE_1 was significantly increased ($p \leq 0.05$) when the cells were exposed to15 mJ/cm^2 compared to irradiated control (FA (-)/UVR(+)) (Figure 6.1B). PGE_1, PGD_1 and 13,14-dihydro-15-keto PGE_1 were slightly decreased following treatment with EPA and post UVB. These changes were not statistically significant compared to the irradiated controls (FA (-)/UVR(+)).

When HaCaT keratinocytes were treated with 10 and 50 µM DHA there was no significant decreased on the level of PGE_1, PGD_1 and 13,14-dihydro-15-keto PGE_1 at baseline when compared to non irradiated control (FA(-)/UVR(-)). However, a significant decreased on PGE1 post UVR was noted when the cells were treated with 10 and 50µM of DHA ($p \leq 0.05$ and 0.01 respectively) compared to irradiated control (FA (-)/UVR(+))(Figure 6.1C,6.2Cand 6.3C).





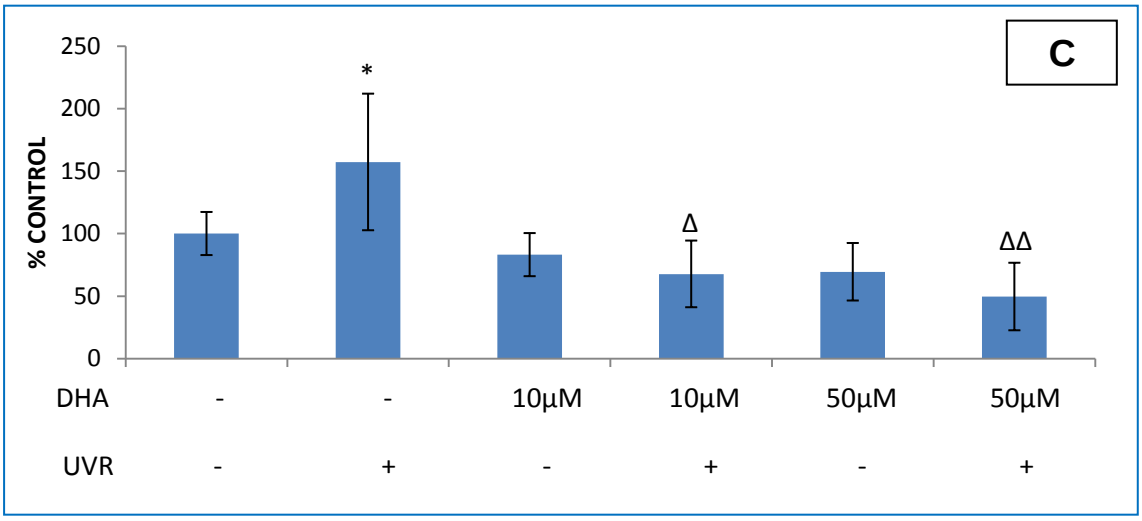
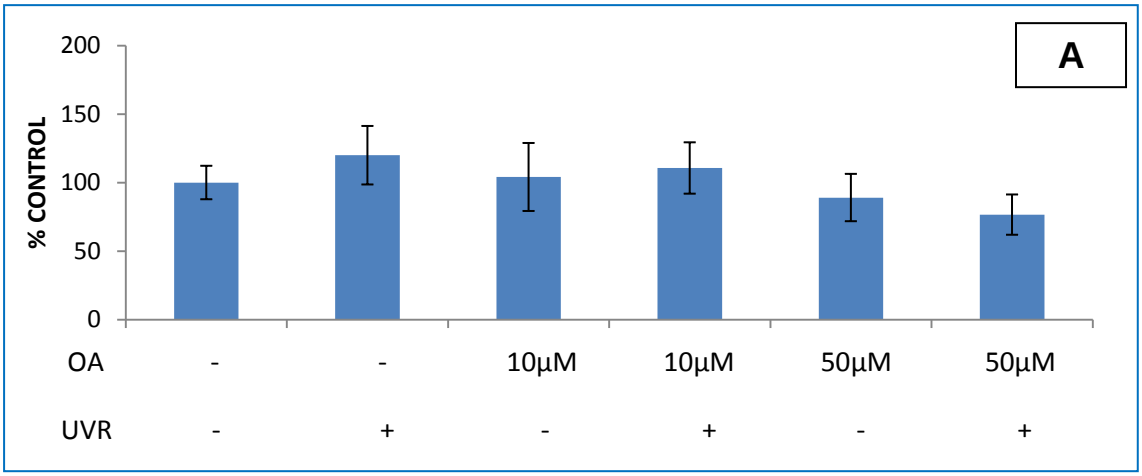
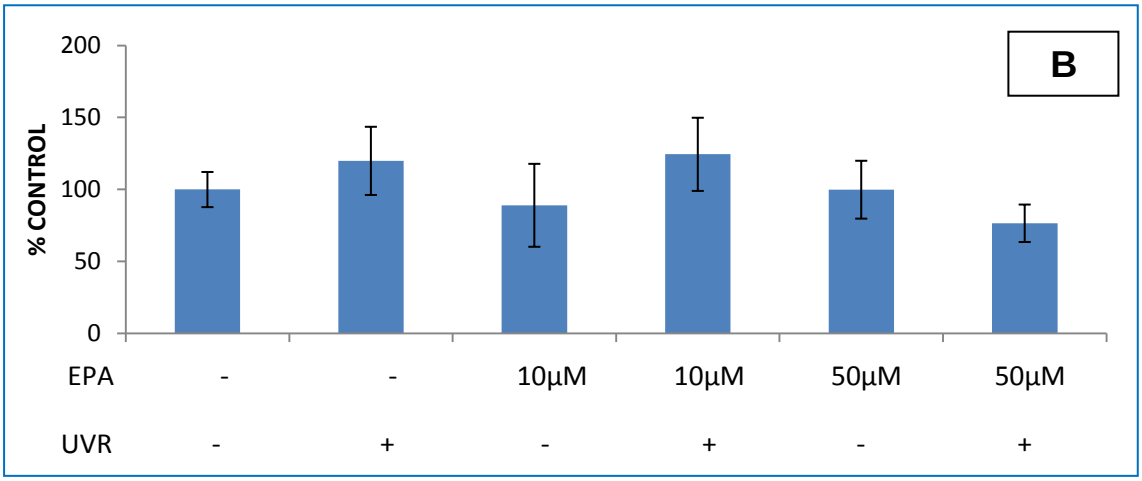


Figure 6.1. Effect of UVR and fatty acid treatment, (A) oleic acid (OA), (B) eicosapentaenoic acid (EPA) and (C) docosahexaenoic acid (DHA) on PGE_1 produced by HaCaT keratinocyte 24 h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *Δp≤0.05, ΔΔp≤0.01, *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).





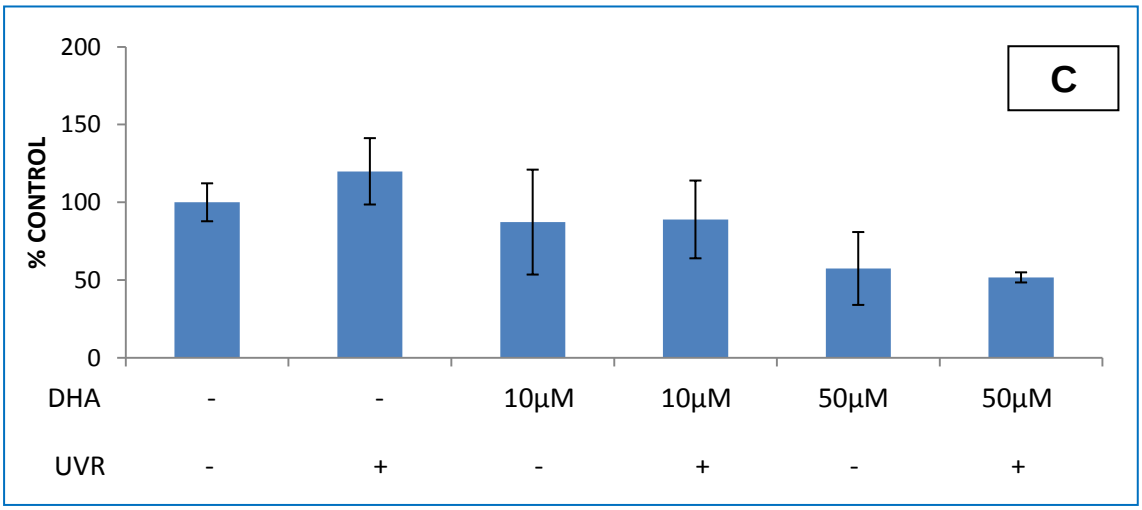


Figure 6.2. Effect of UVR and fatty acid treatment, (A) oleic acid (OA), (B) eicosapentaenoic acid (EPA) and (C) docosahexaenoic acid (DHA) on PGD_1 produced by HaCaT keratinocyte 24 h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate).

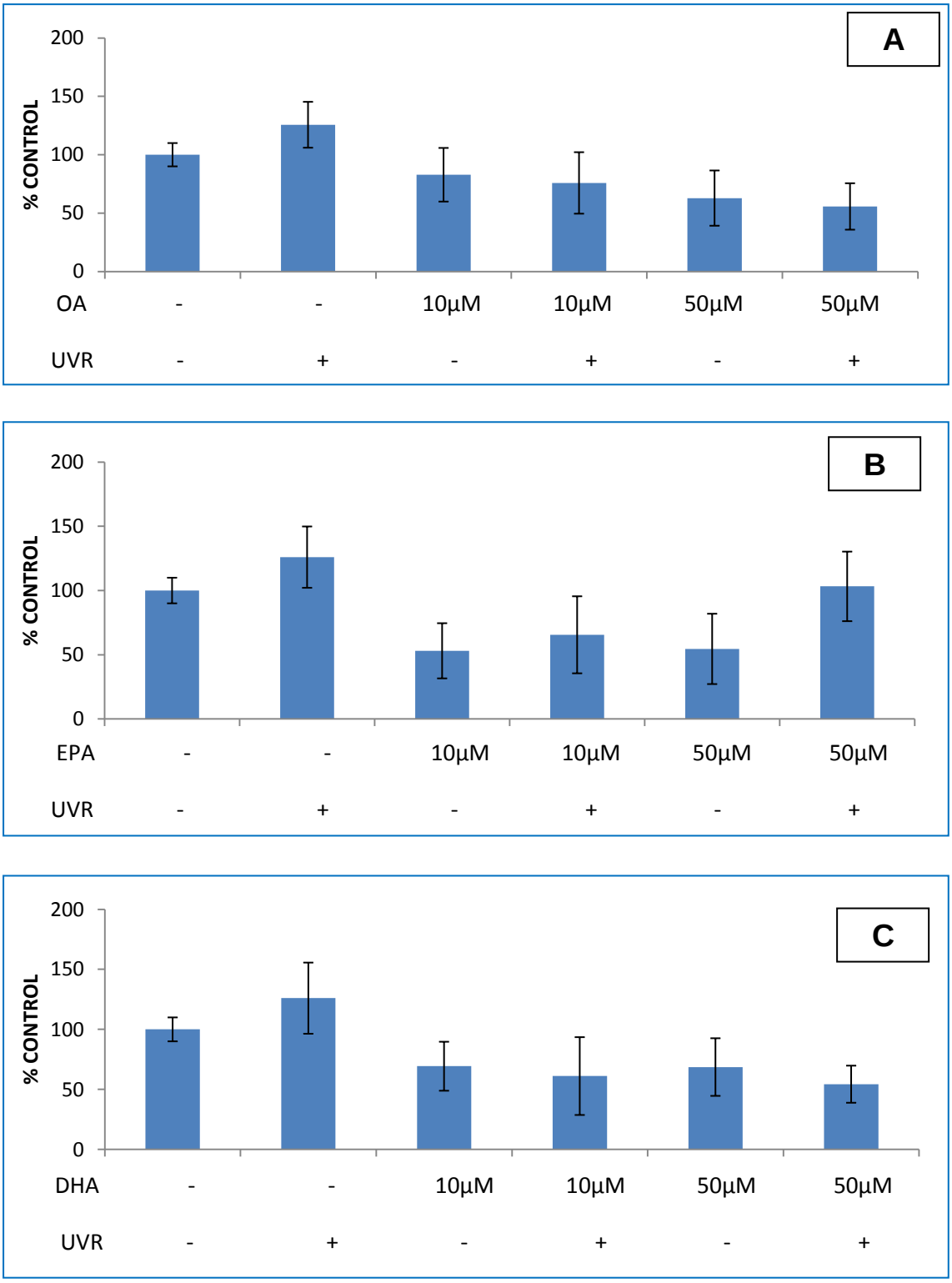


Figure 6.3. Effect of UVR and fatty acid treatment, (A) oleic acid (OA), (C) eicosapentaenoic acid (EPA) and (C) docosahexaenoic acid (DHA)on 13,14-dihydro-15-keto PGE_1 produced by HaCaT keratinocyte 24h post UVR (15 mJ/cm^2). A) OA, B) EPA, C) DHA. Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate).

6.3.2.2. The effect of UVR and fatty acid treatment on series-2 prostanoids

Series-2 prostanoids are products of AA. When HaCaT cells were exposed to 15mJ/cm^2 UVR, we noticed a statistically increased increase in PGE_2 and 13,14-dihydro-15-keto PGE_2 ($p \leq 0.001$and $p \leq 0.05$, respectively) when compared to non irradiated control (FA(-)/UVR(-)) (Figure 6.4 and 6.7). The baseline level of PGE_2, PGD_2, 15-keto PGE_2 and 13,14-dihydro-15-keto PGE_2 did not change when the cells were treated with 10 and 50 µM OA, and compared to non irradiated control (FA(-)/UVR(-)). Results are shown in Figure 6.4A, 6.5A, 6.6A and 6.7A. In addition, OA shown no affect on series 2 prostaglandin levels post UVR compared to irradiated control (FA (-)/UVR(+)).(Figure 6.4A, 6.5A, 6.6A and 6.7A).

The results in Figures 6.4B, 6.5B, 6.6B and 6.7B, show the effect of EPA treatment (10 and 50 µM) on the level of PGE_2, PGD_2, 15-keto PGE_2 and 13,14-dihydro-15-keto PGE_2 produced by HaCaT cells. Although there was an decrease in the level of these mediators when the cells were treated with 10 and 50 µM of EPA, this was not statistically significant when compared to non irradiated control (FA(-)/UVR(-)) (Figures 6.4B, 6.5B, 6.6B and 6.7B). An increase in PGE_2 and 13, 14-dihydro-15-keto PGE_2 was observed when EPA treated HaCaT cells were exposed to 15mJ/cm^2 UVR, and this was statistically significant ($p \leq 0.001$and $p \leq 0.05$, respectively) compared to non-irradiated control (FA(-)/UVR(-)) Figure 6.4B and 6.7B. The levels of PGD_2 and 15-keto PGE_2 were increased when the cells were exposed to with 15m J/cm^2, but this change was not statistically significant when compared to non-irradiated control (FA(-)/UVR(-)) (Figure 6.5B and 6.6B). HaCaT treated with 10 and 50 µM of EPA and exposed to 15 mJ/cm^2 UVR showed a significant decreased in PGE_2 ($p \leq 0.05$

and p≤0.01, respectively) and 13,14-dihydro-15-keto PGE_2 production (p≤0.05 and p≤0.01, respectively), but changes in PGD_2, and 15-keto PGE_2 were not significant.(Figure 6.4B, 6.5B, 6.6B and 6.7B).

HaCaT treated cells DHA with (10 µM) showed no statistically significant changes in PGE_2, PGD_2, 15-keto PGE_2 and 13,14-dihydro-15-keto PGE_2 productionwhen compared to non irradiated controls (FA(-)/UVR(-)). Results shown in Figures 6.4C, 6.5C, 6.6C and 6.7C. However, ssignificantly reduced level of PGE_2 (p≤0.05) and 13,14-dihydro-15-keto PGE_2 (p≤0.05) was observed when the cells were treated with 50 µM DHA. Exposure of untreated HaCaT cells to 15 mJ/cm^2 showed significant increase in PGE_2 and 13,14-dihydro-15-keto PGE_2 (p≤0.001 and p≤0.05, respectively) when compared to the corresponding controls (FA(-)/UVR(-))(Figure 6.4C and 6.7C). Whilst, no significant increase in HaCaT cell PGD_2 and 15-keto PGE_2 were observed. (Result shown in Figure 6.5C and 6.6C). As shown in Figures 6.4C and 6.7C, DHA induced a dose-dependent reduction in PGE_2 and 13,14-dihydro-15-keto PGE_2 levels post exposure to 15 mJ/cm^2. DHA (10 µM) significantly reduced the levels of PGE_2 (p≤ 0.01) and 13,14-dihydro-15-keto PGE_2 (p≤0.001) compared to irradiated control cells (FA(-)/UVR(-)). The of DHA (50 µM) treatment further reduced the concentration of PGE_2 (p≤0.01) and 13,14-dihydro-15-keto PGE_2 (p≤0.001) compared to irradiated control cells (FA(-)/UVR(+)). However, the reduction in the levels of PGD_2 and 15-keto PGE_2 post UVR were not statistically significant when compared to irradiated control (FA (-)/UVR(+)). (Figure 6.5C and 6.6C).

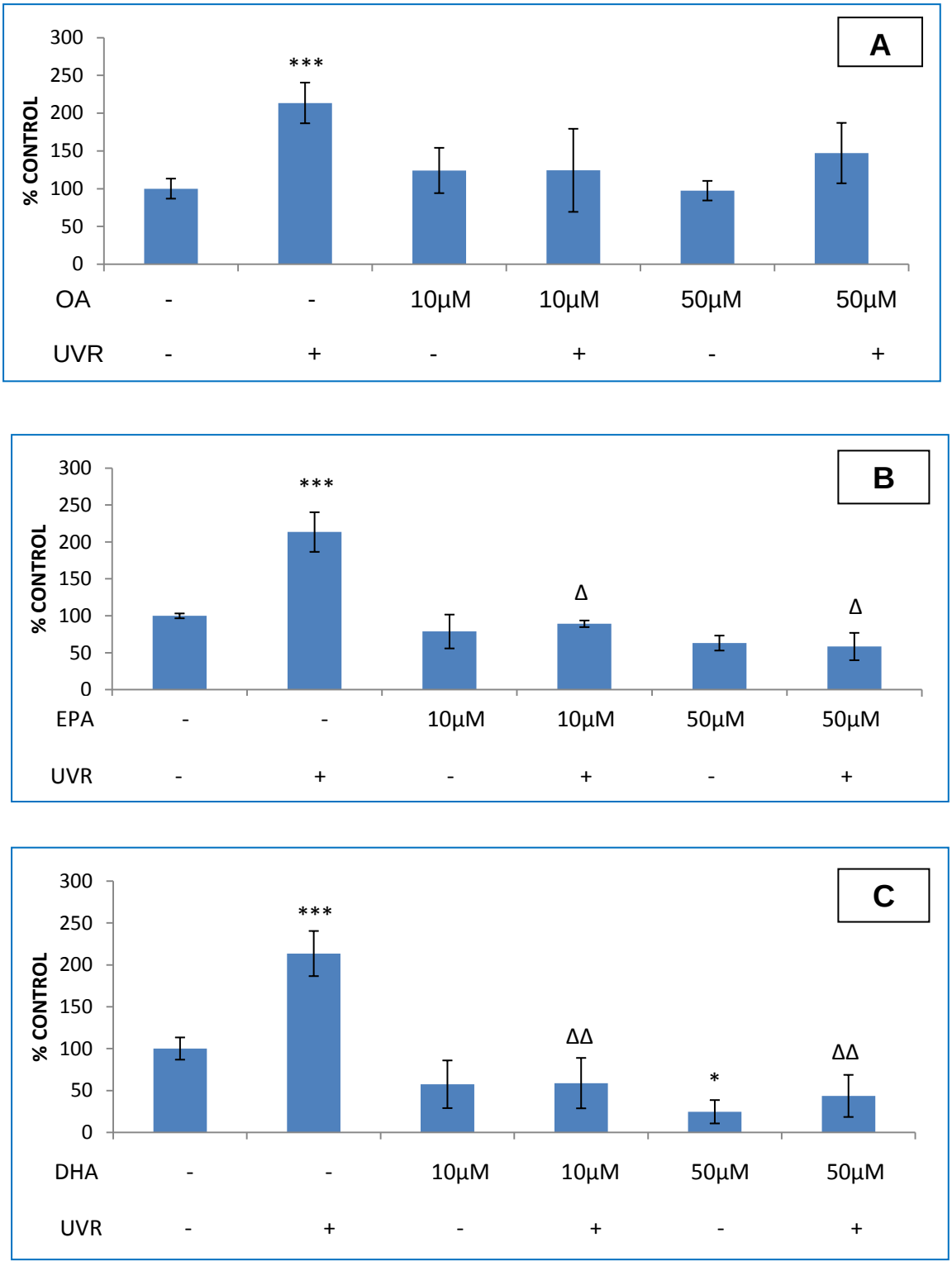


Figure 6.4. Effect of fatty acid and UVR treatment, (A) oleic acid (OA), (B) eicosapentaenoic acid (EPA) and (C) docosahexaenoic acid (DHA) on PGE_2 produced by HaCaT keratinocyte 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *Δp≤0.05, **ΔΔp≤0.01 ***,ΔΔΔp≤0.001, *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

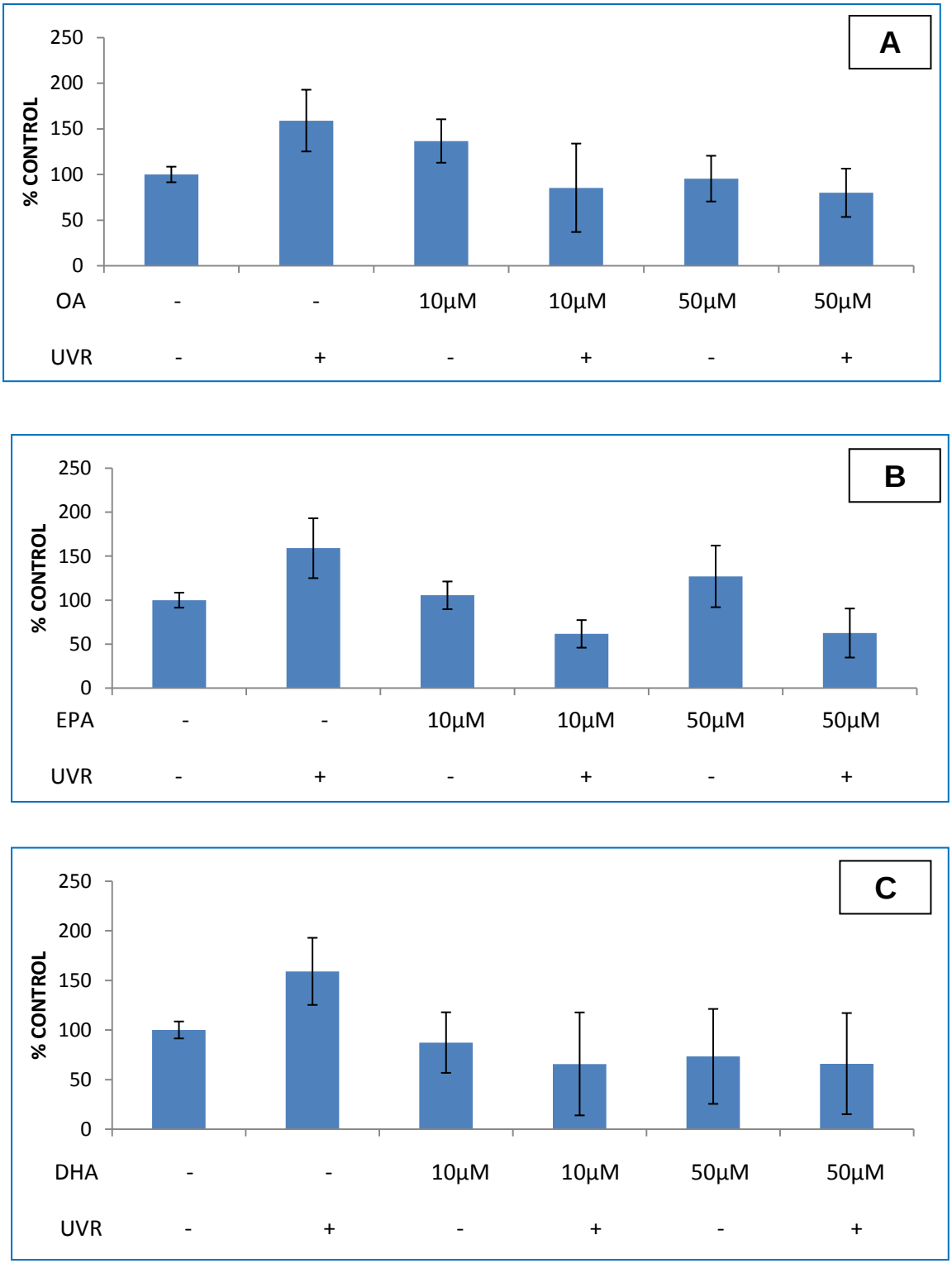


Figure 6.5. Effect of UVR and fatty acid treatment, (A) oleic acid (OA), (B) eicosapentaenoic acid (EPA) and (C) docosahexaenoic acid (DHA) on PGD_2 produced by HaCaT keratinocyte 24 h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate).

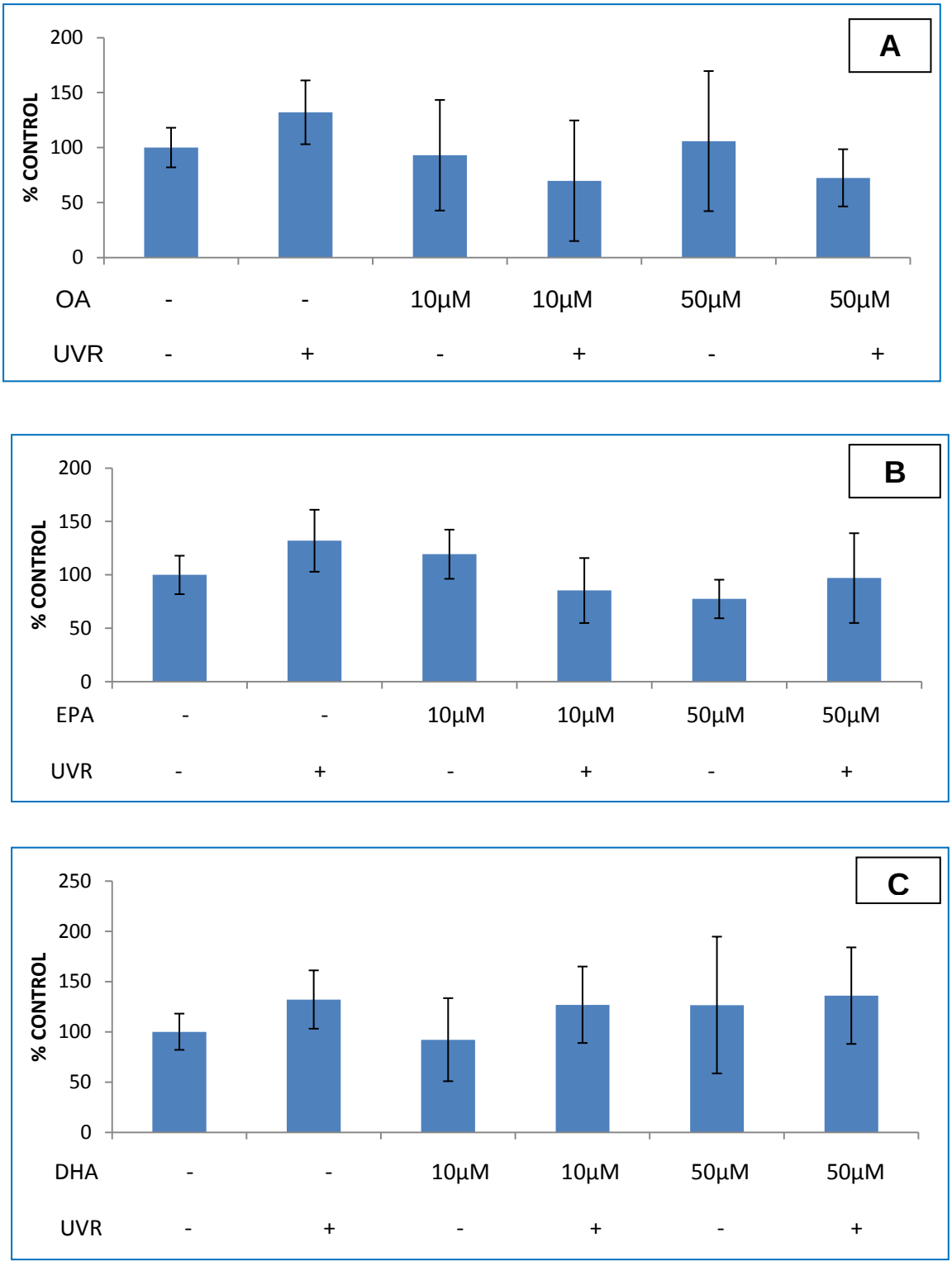


Figure 6.6. Effect of UVR and fatty acid treatment, (A) oleic acid (OA), (B) eicosapentaenoic acid (EPA) and (C) docosahexaenoic acid (DHA)on 15-keto PGE_2 produced by HaCaT keratinocyte 24 h post UVR (15 mJ/cm^2). A) OA, B) EPA, C) DHA. Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate).

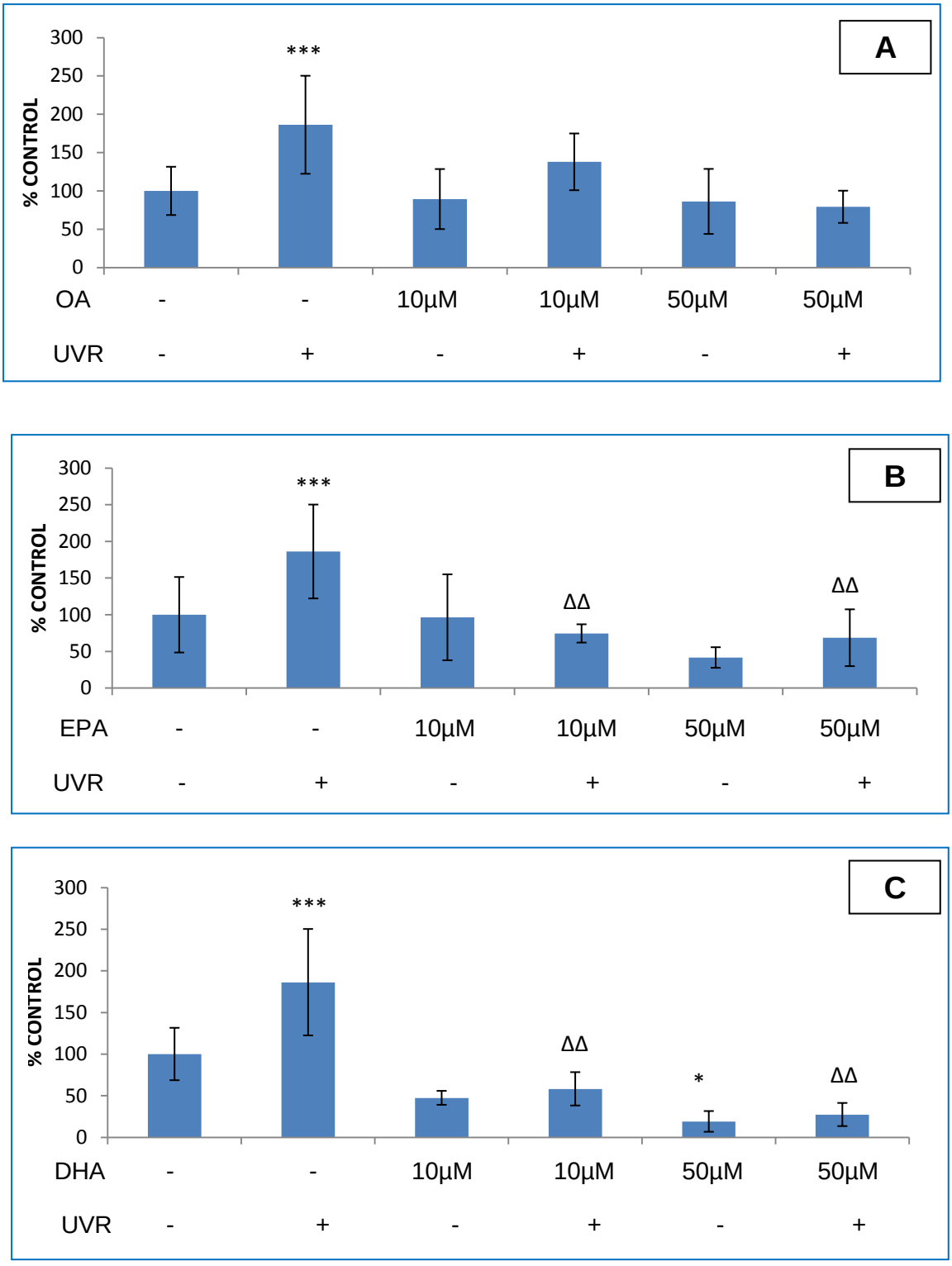


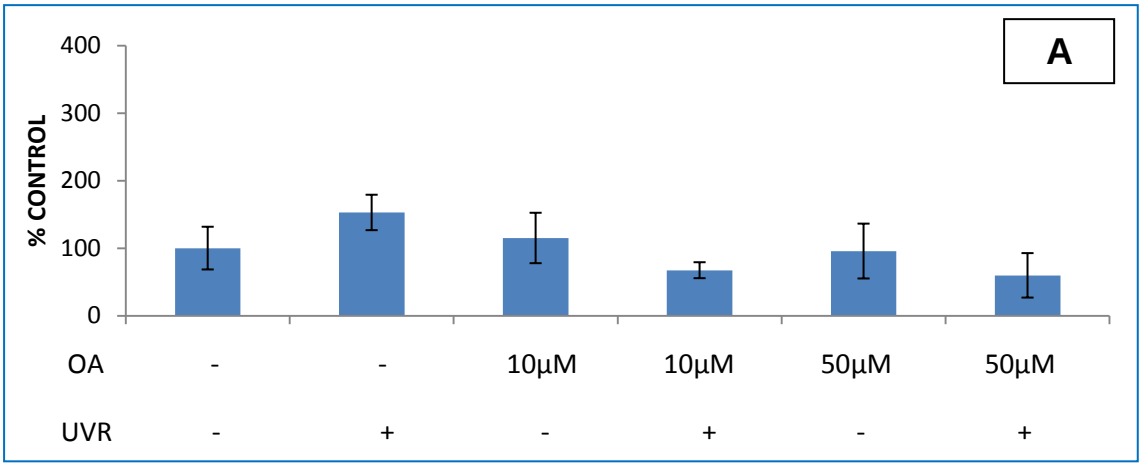
Figure 6.7. Effect of UVR and fatty acid treatment, oleic acid (OA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)treatment on HaCaT 13,14-dihydro-15-keto PGE_2 produced by HaCaT keratinocyte 24 h post UVR (15 mJ/cm2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *Δp≤0.05, **ΔΔp≤0.01 ***,ΔΔΔp≤0.001, *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

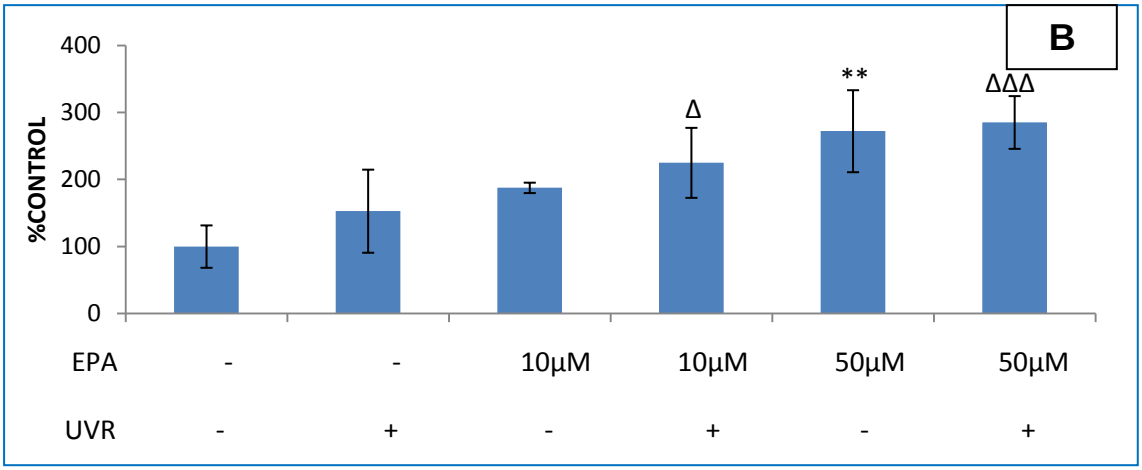
6.3.2.3. The effect of UVR and fatty acid treatment on series-3 prostanoids

Exposure of untreated HaCaT cells to 15mJ/cm^2 showed no significant increase in the EPA products PGE_3 and PGD_3 when compared to the corresponding controls (FA(-)/UVR(-)). Figures 6.8A and 6.9A show the effect of OA treatment on PGE_3 and PGD_3. There was no a significant deference in PGE_3 and PGD_3 levels when HaCaT cells were treated with 10 and 50 µM of OA and compared to non-treated cells. Also, there was no significant deferent on PGE_3 and PGD_3 levels when HaCaT cells treated with 10 and 50 µM of OA, were exposed to 15 mJ/cm^2.

We did not observe any statistically significant increase on PGE_3 and PGD_3 levels when HaCaT cells were treated with 10 µM of EPA, compared to the corresponding controls (FA(-)/UVR(-)) (Figure 6.8B and 6.9B). But when the cells were treated with EPA (50 µM) a shown significant increase ($p \leq 0.01$) in PGE_3 but not in PGD_3 was noted. (Figure 6.8B and 6.9B). In order to see the effect of EPA on PGE_3 and PGD_3 post UVR, EPA treated irradiated cells were compared to untreated irradiated cells (FA(-)/UVR (+)). The results shown in Figure 6.8 C show that EPA at 10 and 50 µM has significant effect on PGE_3 ($p \leq 0.05$ and $p \leq 0.001$) not in PGD_3.

Finally, when cells were treated with DHA (10 and 50 µM), PGE_3 and PGD_3 levels were not statistically deferent when compared to the corresponding controls (FA(-)/UVR(-)) (Figure 6.8C and 6.9C). Also, there was no significant difference on the levels of PGE_3 and PGD_3 produced by DHA treated with HaCaT cell when exposed to 15 mJ/cm^2 UVR results showed in figures 6.8C and 6.9C). A summary of the effect of UVR and n-3 PUFA on series-1, -2 and -3 prostanoids are shown in Table (6.2).





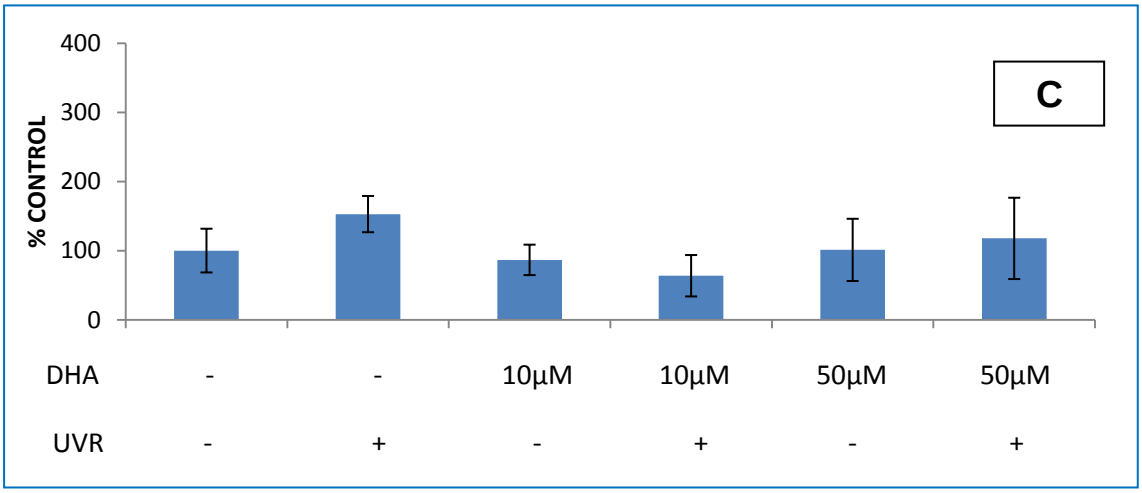


Figure 6.8. Effect of UVR and fatty acid treatment, oleic acid (OA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on PGE_3 produced by HaCaT keratinocyte 24 h post UVR (15 mJ/cm2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *Δp≤0.05, **ΔΔp≤0.01 ***,ΔΔΔp≤0.001, *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

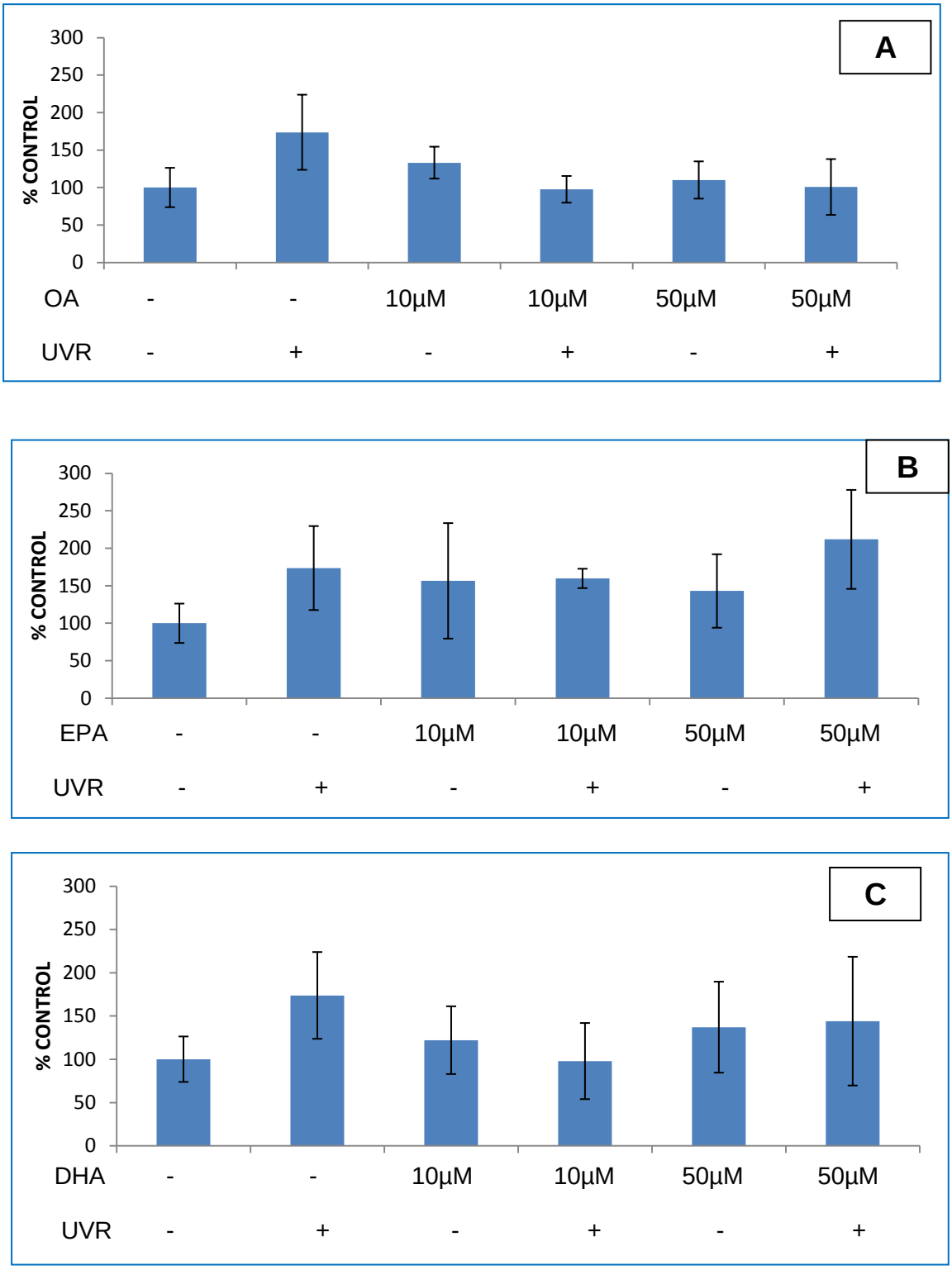


Figure 6.9. Effect of UVR and fatty acid treatment, oleic acid (OA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on PGD_3 produced by HaCaT keratinocyte 24 h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate).

Table 6.2. Summary of the effect of UVR and n-3 PUFA treatment on the prostanoid mediators produced by HaCaT keratinocyte

	UVR (15mJ/cm^2)	No UVR		UVR (15mJ/cm^2)	
Compound		**EPA**	**DHA**	**EPA**	**DHA**
PGE_1	↑*	—	—	—	↓Δ
PGD_1	—	—	—	—	—
PGE_2	↑*	—	↓*	↓Δ	↓Δ
PGD_2	—	—	—	—	—
PGE_3	—	↑*	—	↑Δ	—
PGD_3	—	—	—	—	—
15-keto PGE_2	—	—	—	—	—
13,14dihydro-15-PGE_1	—	—	—	—	—
13,14dihydro-15-PGE_2	↑*	—	↓*	↓Δ	↓Δ

—= No significant effect

↓= Significant decrease

↑= Significant increase

*= compared to untreated cells (FA(-)/UVR (-))

Δ= compared to untreated irradiated cells (FA(-)/UVR (+))

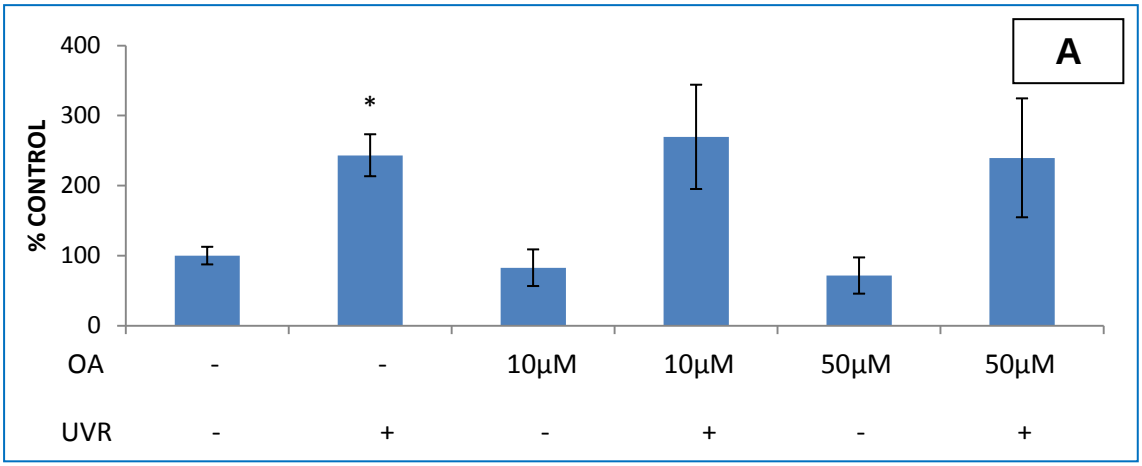
6.3.3. The effect of UVR and fatty acid treatment on prostanoids produced by 46BR.1N cells

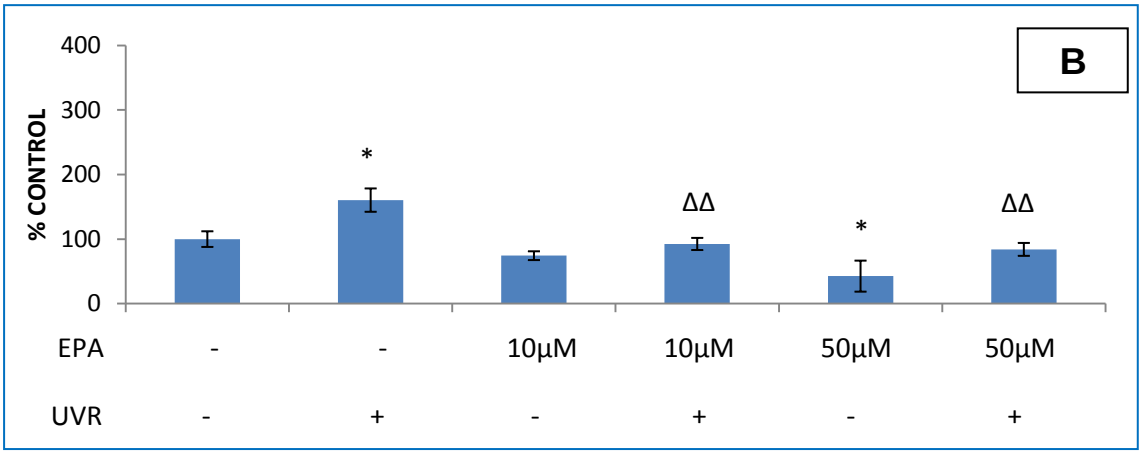
6.3.3.1. The effect of UVR and fatty acid treatment on series-1 prostanoids

PGE_1, PGD_1 and 13,14-dihydro-15-keto PGE_1 levels were increased post UVR exposure, with the increases in PGE_1 and 13,14-dihydro-15-keto PGE_1 being statistically significant ($p \leq 0.01$ and $p \leq 0.001$, respectively) compared to non-irradiated control (FA(-)/UVR(-)). Figures 6.10A, 6.11A and 6.12A show the effect of OA treatment on 46BR.1N PGE_1, PGD_1 and 13,14-dihydeo-15-keto PGE_1. There were no significant changes in the levels of these mediators when cells were treated with 10 and 50µM of OA or when the OA treated cells were exposed to UVR compared to irradiated control (FA (-)/UVR(+)).

There was no significant decrease on PGE1, PGD_1 and 13,14-dihydeo-15-keto PGE_1 when 46BR.1N cells were treated with 10 µM of EPA. But a significant decrease ($p \leq 0.05$) was noted for PGE_1 when 46BR.1N cells were treated with 50 µM EPA compared to control (Figures 6.10B, 6.11B and 6.12B). Furthermore, the levels of PGE_1, PGD_1 and 13,14-dihydeo-15-keto PGE_1 in EPA treated cells increased post UVR. But only PGE_1 was significantly lower post 10 ($p \leq 0.01$) and 50µM EPA ($p \leq 0.01$) (Figures 6.10B, 6.11B and 6.12B)

Finally, when cells were treated with 10 and 50 µM DHA the levels of PGE_1, PGD_1 and 13,14-dihydeo-15-keto PGE_1 did not change compared to non irradiated controls. Although, the DHA treated appeared to reduce the levels of PGE_1, PGD_1 and 13,14-dihydeo-15-keto PGE_1 post UVR, none of the observed changes was statistically significant compared to irradiated controls (FA (-)/UVR(+))(Figure 6.10C, 6.11C and 6.12C).





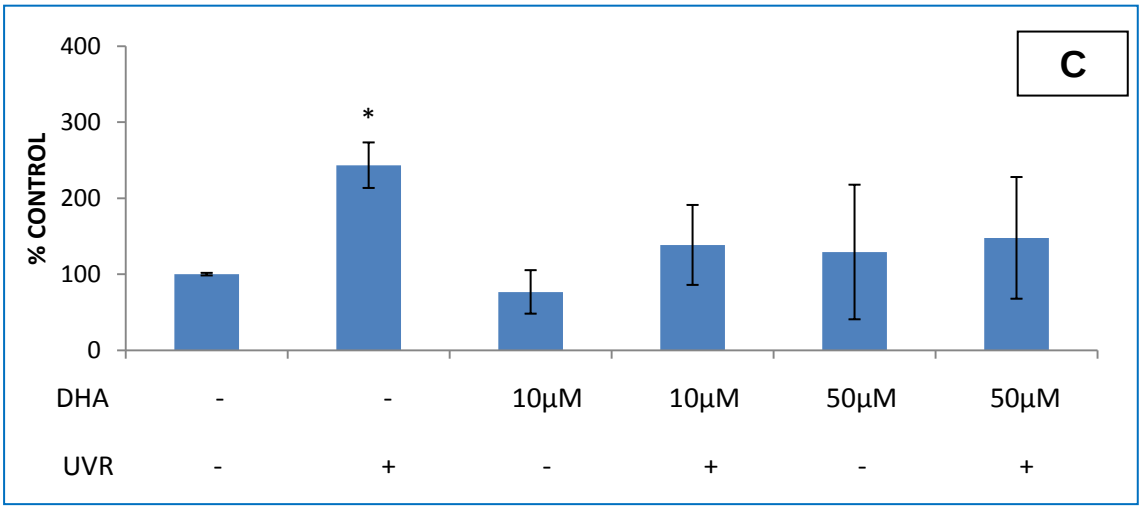
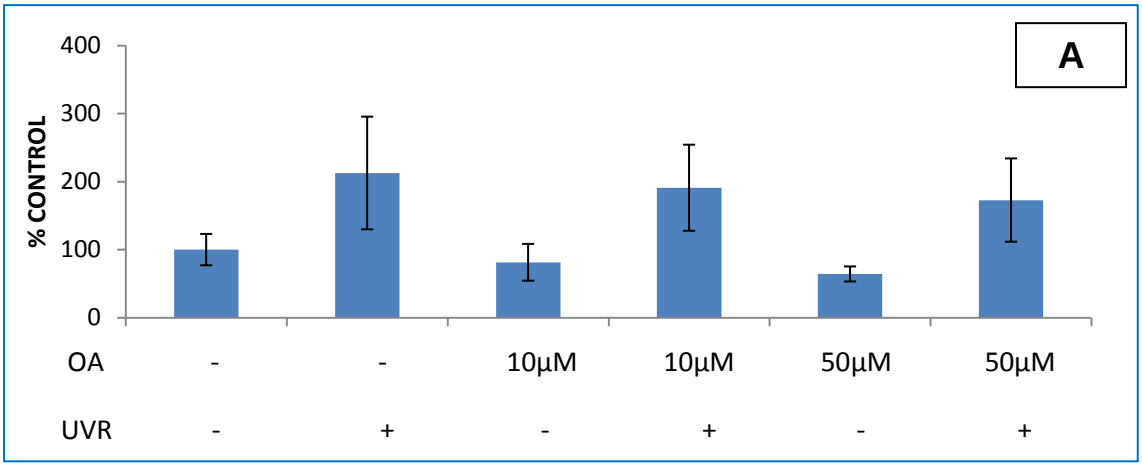
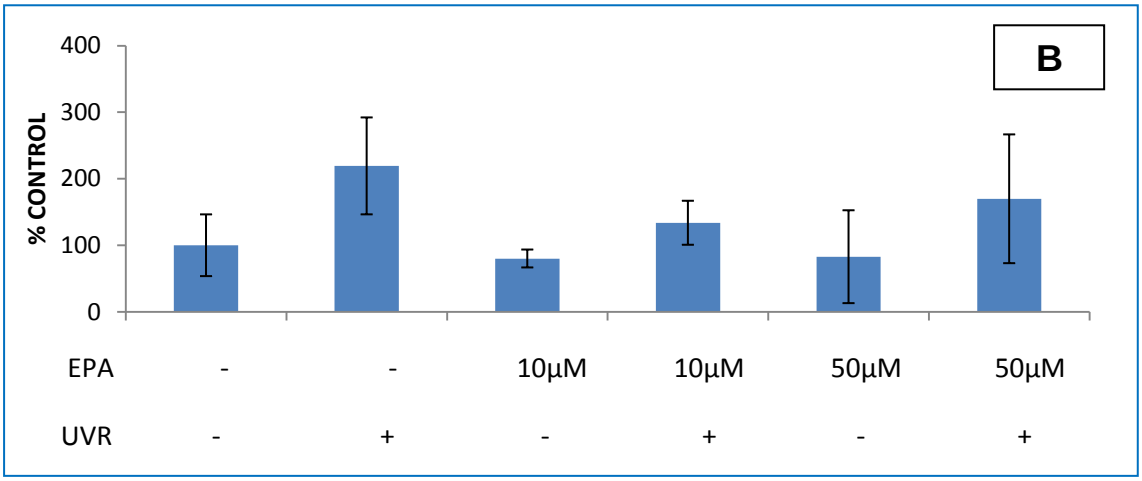


Figure 6.10. Effect of UVR and fatty acid treatment, (A) oleic acid (OA), (B) eicosapentaenoic acid (EPA) and (C) docosahexaenoic acid (DHA)on PGE_1 produced by 46BR.1N fibroblasts 24 h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *Δp≤0.05, **ΔΔp≤0.01, *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).





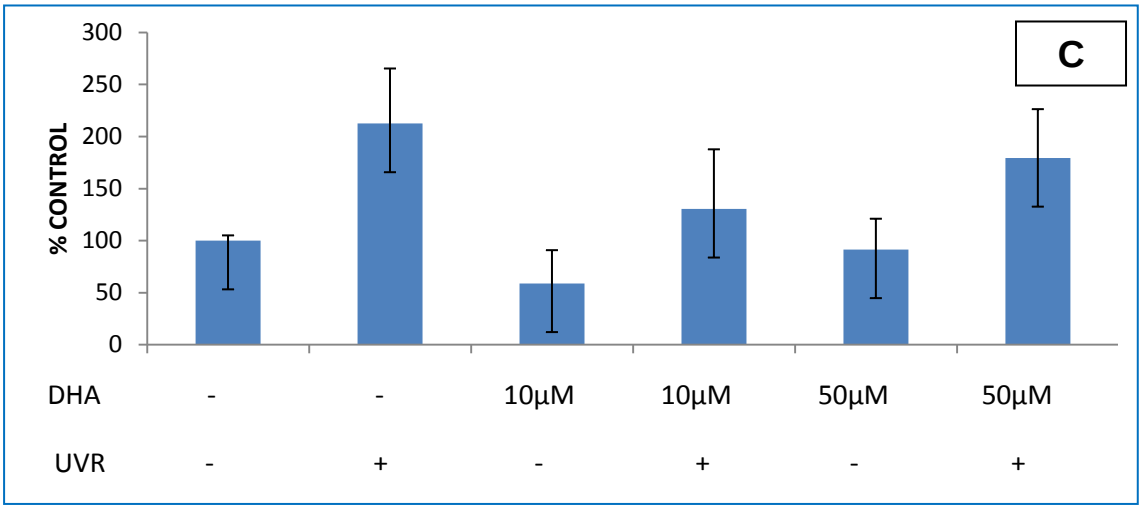


Figure 6.11. Effect of UVR and fatty acid treatment, (A) oleic acid (OA), (B) eicosapentaenoic acid (EPA) and (C) docosahexaenoic acid (DHA) on PGD_1 produced by 46BR.1N fibroblasts 24 h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate).

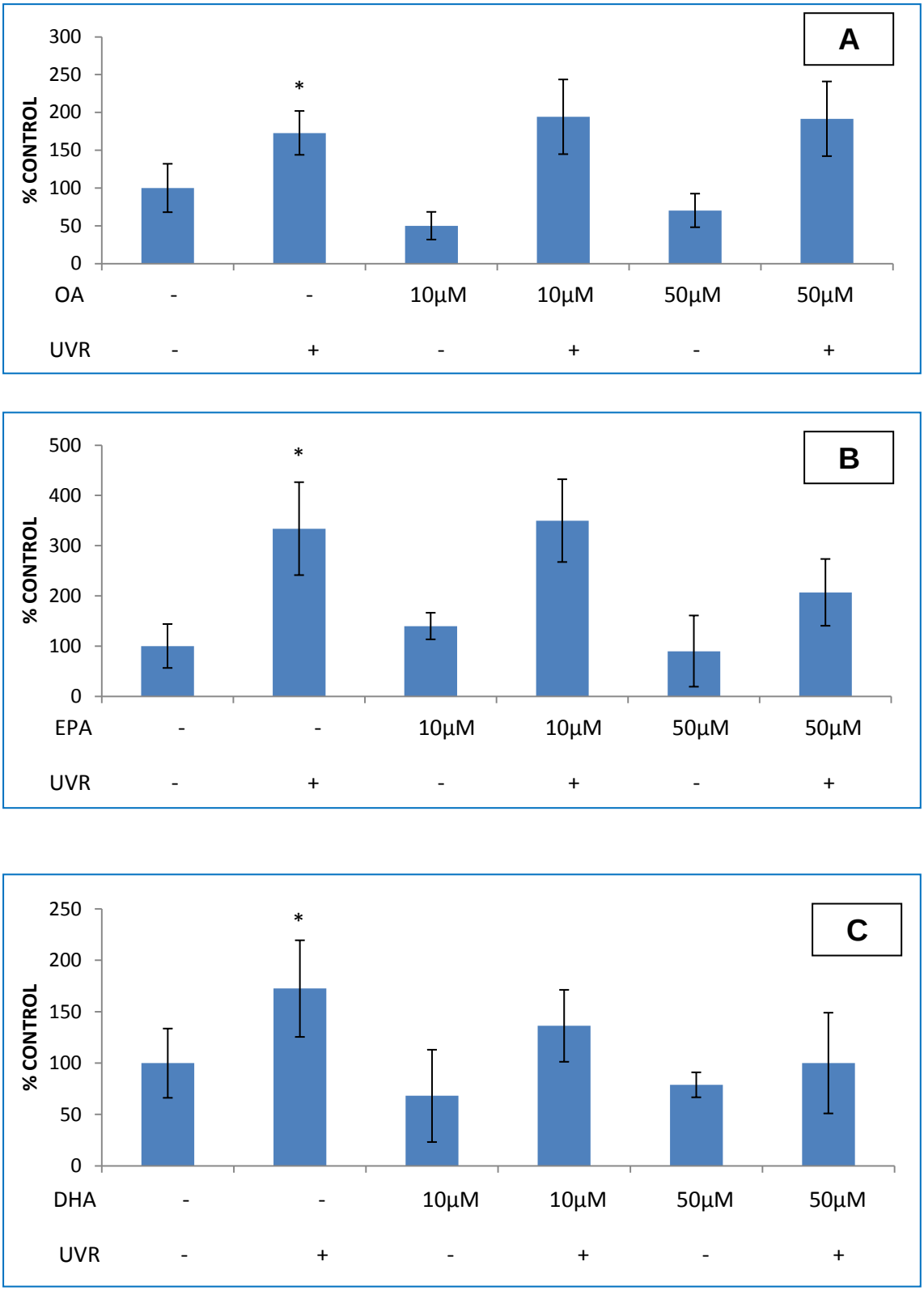


Figure 6.12. Effect of UVR and fatty acid treatment, (A) oleic acid (OA), (B) eicosapentaenoic acid (EPA) and (C) docosahexaenoic acid (DHA)on 13,14-dihydro-15-keto PGE_1 produced by 46BR.1N fibroblasts 24 h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *p≤0.05, *=compared to non-irradiated control (FA(-)/UVR(-)).

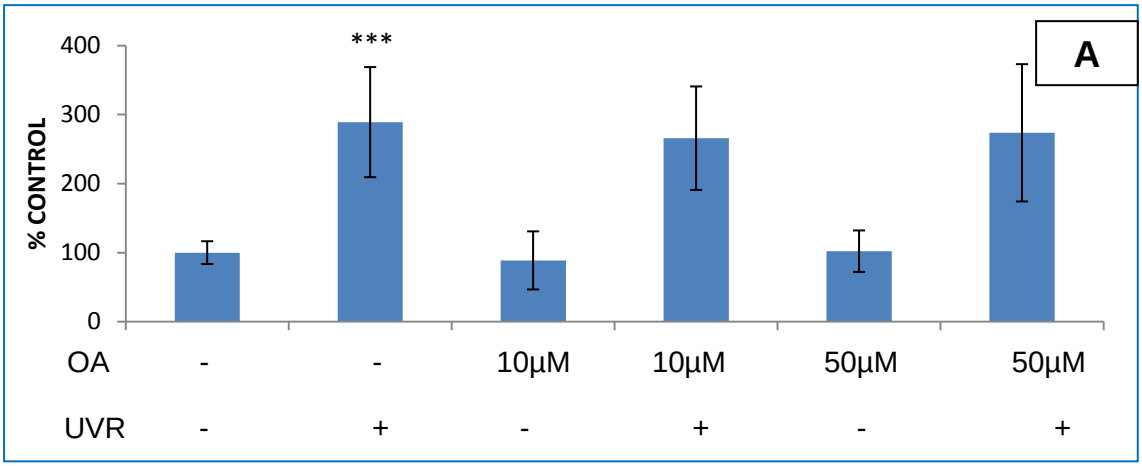
6.3.3.2. The effect of UVR and fatty acid treatment on series-2 prostanoids

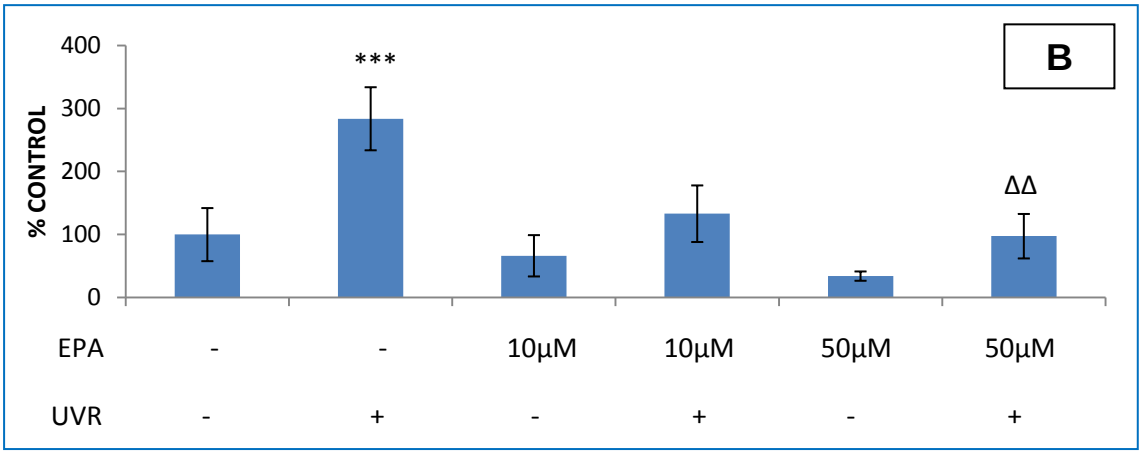
When 46BR.1N cells were exposed to 15mJ/cm^2 UVR, the levels of PGE_2 and 15-keto PGE_2 were found to be statistically significantly higher ($p \leq 0.001$ and $p \leq 0.05$; respectively) when compared to non irradiated controls (FA(-)/UVR(-)) (Figures 6.13 and 6.15). The levels of PGE_2, PGD_2, 15-keto PGE_2 and 13,14-dihydro-15-keto PGE_2 did not change when cells were treated with 10 and 50 µM OA and compared to non irradiated control (FA(-)/UVR(-)). Result shown in Figure 6.13A, 6.14A, 6.15A and 6.16. In addition, OA treatment did not have any statistically significant on series 2 prostaglandins levels post UVR when compared to irradiated control (FA (-)/UVR(+))(Figure 6.13A, 6.14A, 6.15A and 6.16).

Figures 6.13B, 6.14B, 6.15B and 6.16B show the effect of EPA treatment (10 and 50 µM) on PGE_2, PGD_2, 15-keto PGE_2 and 13,14-dihydro-15-keto PGE_2 reduced by 46BR.1N cell level. Although there was an decrease in the level of this mediators when the cells were treated with 10 and 50 µM of EPA, this was not statistically significant when compared to non irradiated control (FA(-)/UVR(-)). Also, the levels of PGD_2, 15-keto PGE_2 and 13,14-dihydro-15-keto PGE_2 were decreased when 46BR.1N cells were treated with 10 and 50 µM of EPA and exposed to 15 mJ/cm^2 UVR, these changes were not statistically significant when compared to irradiated control (FA (-)/UVR(+)). (Figure 6.14B, 6.15B and 6.16B). As shown in Figure 6.13B, EPA (50 µM) significantly reduced PGE_2 levels ($p \leq 0.05$) after 46BR.1N cells were exposed to 15 mJ/cm^2 and compared to irradiated control (FA (-)/UVR(+)).

The production of PGE_2, PGD_2, 15-keto PGE_2 and 13,14-dihydro-15-keto PGE_2 by 10 and 50 µM DHA treated 46BR.1N cells showed no statistically significant change when compared to the non irradiated control (FA(-)/UVR(-)). (Results shown in figure 6.13C, 6.14C, 6.15C and 6.16C).

As shown in Figure 6.13C, DHA induced a dose-dependent reduction in PGE_2 level in the 46BR.1N cells after they were exposed to 15 mJ/cm^2. DHA (10 µM) significantly reduced PGE_2 levels ($p \leq 0.01$) compared to irradiated control cells (FA(-)/UVR(-)) and 50 µM of DHA reduced the concentration of PGE_2 even more and this was statistically significant ($p \leq 0.001$) compared to irradiated control cells (FA(-)/UVR(+)). Although, the DHA treatment reduced the level of PGD_2 and 13,14-dihydro-15-keto PGE_2 post UVR, but these changes were not statistically significant when compared to irradiated control (FA (-)/UVR(+)) (Figure 6.14C and 6.16C).





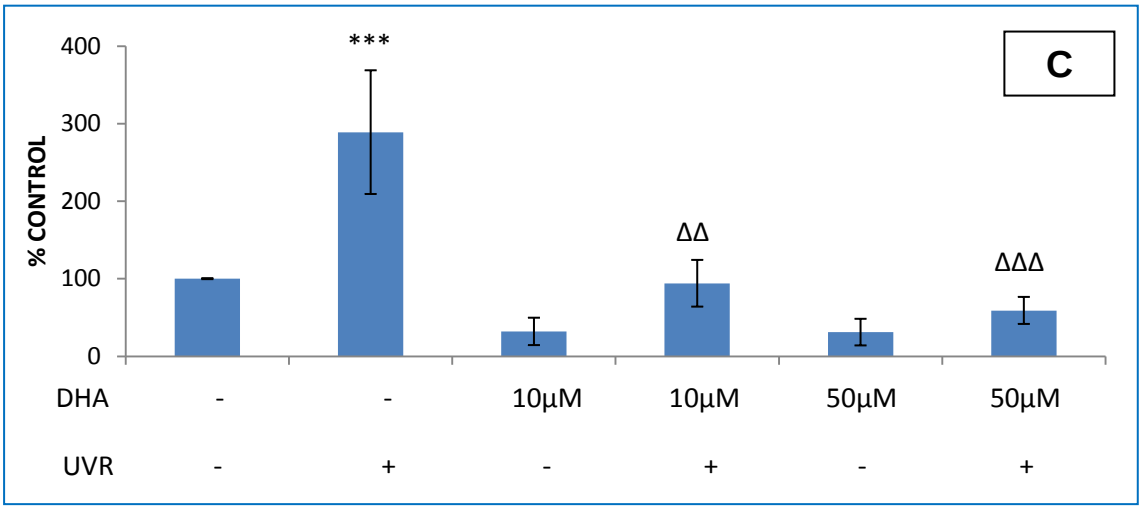
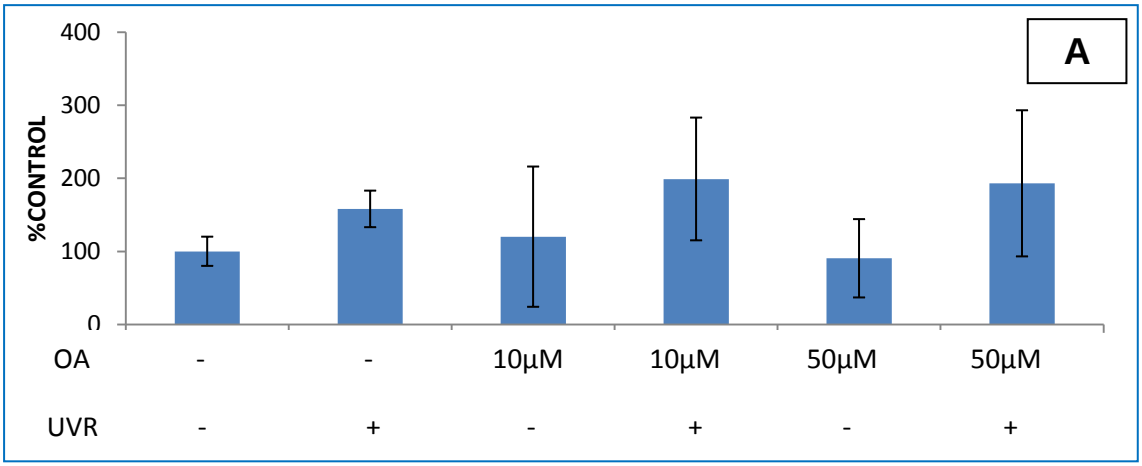
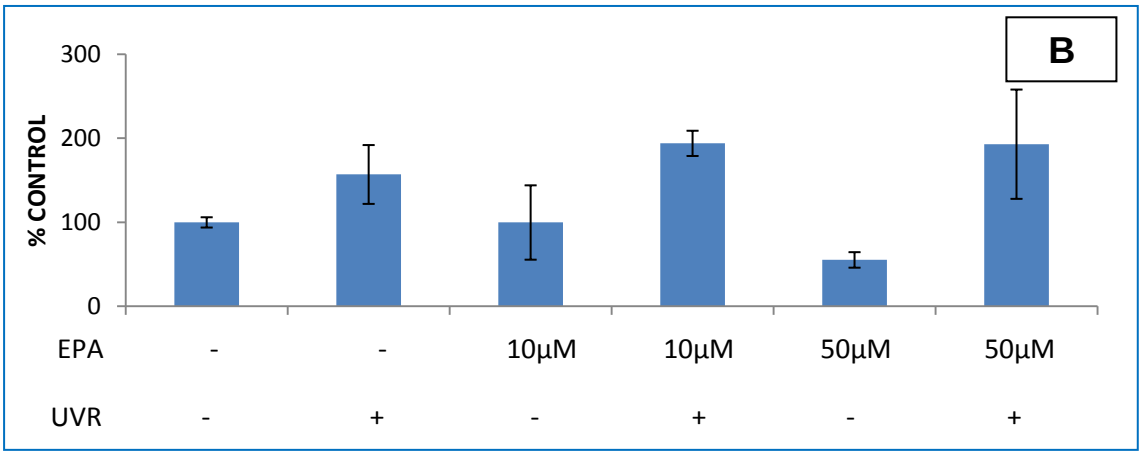


Figure 6.13. Effect of UVR and fatty acid treatment, (A) oleic acid (OA), (B) eicosapentaenoic acid (EPA) and (C) docosahexaenoic acid (DHA) on PGE_2 produced by 46BR.1N fibroblasts 24 h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). ΔΔp≤0.01 ***,ΔΔΔp≤0.001, *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).





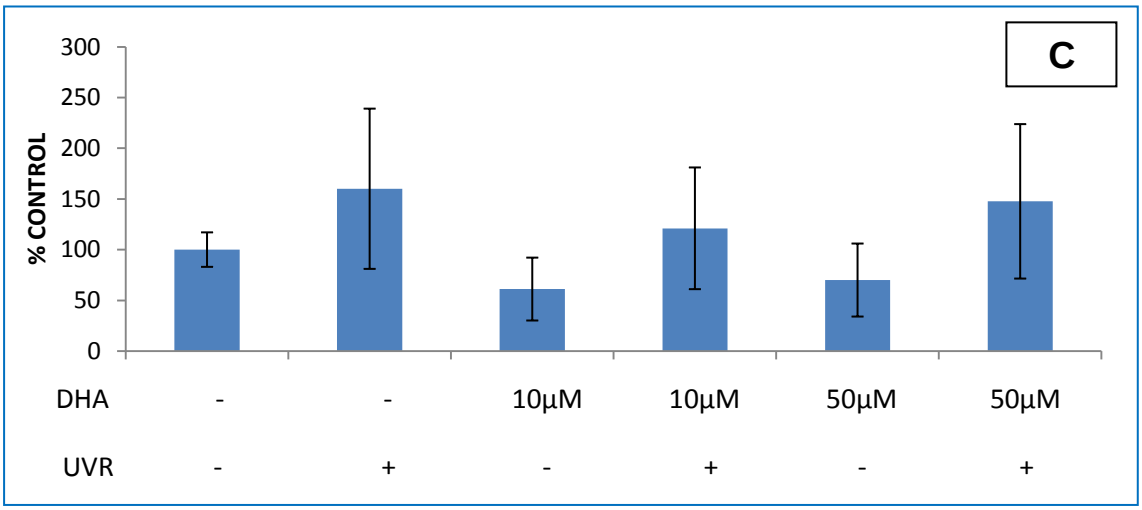


Figure 6.14. Effect of UVR and fatty acid treatment, (A) oleic acid (OA), (B) eicosapentaenoic acid (EPA) and (C) docosahexaenoic acid (DHA)on 46BR.1N PGD_2 produced by 46BR.1N fibroblasts 24 h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate).

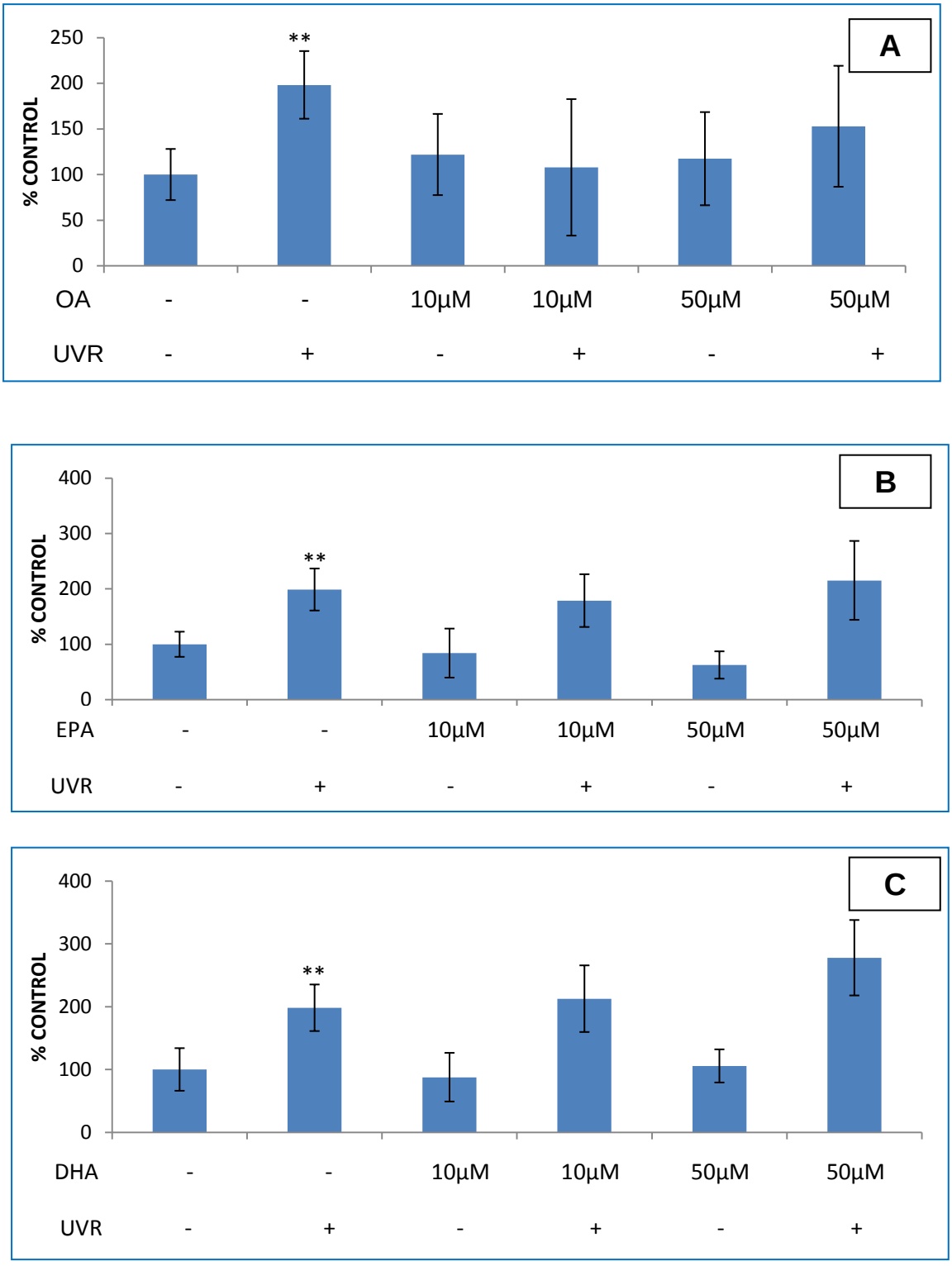
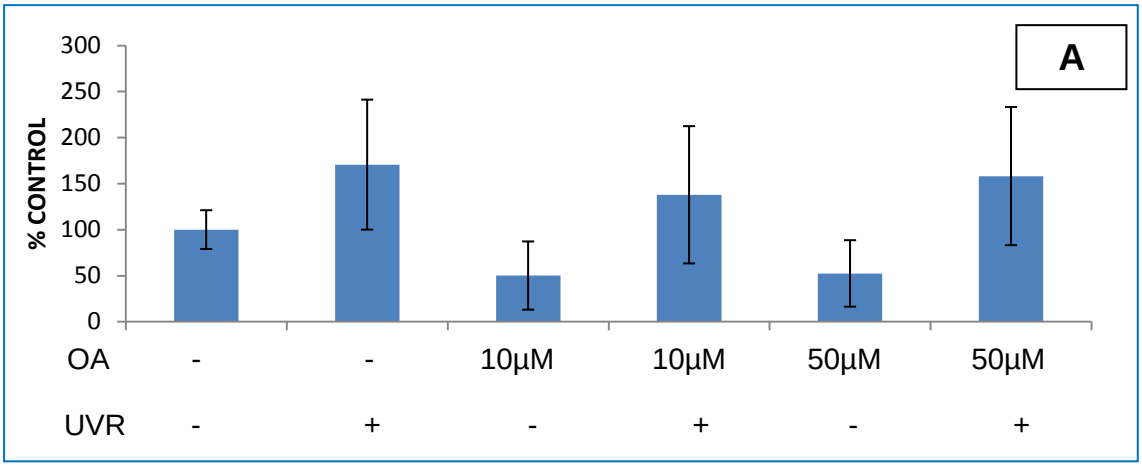
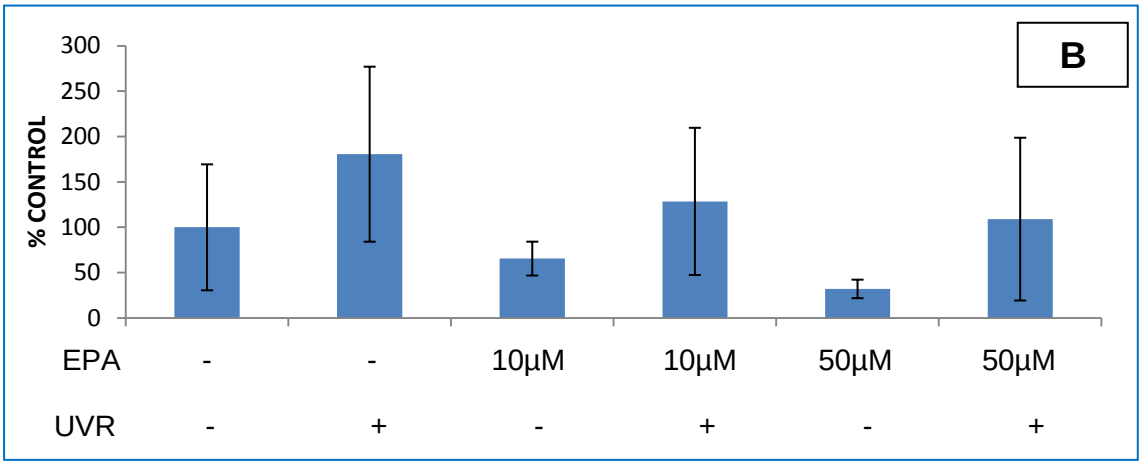


Figure 6.15. Effect of UVR and fatty acid treatment, (A) oleic acid (OA), (B) eicosapentaenoic acid (EPA) and (C) docosahexaenoic acid (DHA) on 46BR.1N 15-keto PGE_2 produced by 46BR.1N fibroblasts 24 h post UVR (15 mJ/cm^2). A) OA, B) EPA, C) DHA. Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). **p≤0.01,*=compared to non-irradiated control (FA(-)/UVR(-)).





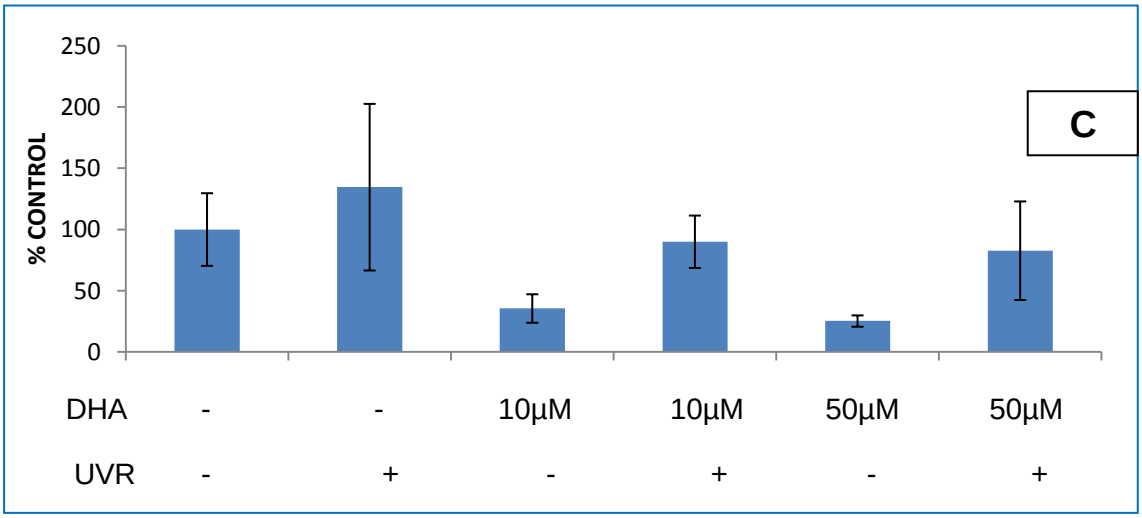


Figure 6.16. Effect of UVR and fatty acid treatment, (A) oleic acid (OA), (B) eicosapentaenoic acid (EPA) and (C) docosahexaenoic acid (DHA) on 13,14-dihydro-15-keto PGE_2 produced by 46BR.1N fibroblasts 24 h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate).

6.3.3.3. The effect of UVR and fatty acid treatment on series-3 prostanoids

Exposure of 46BR.1N cells to 15 mJ/cm^2 showed significant increases in PGE_3 ($p \leq 0.01$) levels but the increase in PGD_3 failed to reach statistical significances when compared to the negative controls (FA(-)/UVR(-)). Figures 6.17A and 6.18A shows the effect of OA treatment on PGE_3 and PGD_3. There were no significant differences on PGE_3 and PGD_3 levels when the 46BR.1N cells were treated with 10 and 50 µM OA and when exposed to 15 mJ/cm^2.

Although there was no significant increase on PGE_3 and PGD_3 when 46BR.1N cells were treated with 10 µM of EPA, higher concentration of EPA (50 µM) increased the levels of PGE_3 but not PGD_3 and this was significant ($p \leq 0.001$) compared to the corresponding controls (FA(-)/UVR(-)). (data shown in figure 6.17B and 6.18B). In order to see if EPA have an effects on PGE_3 and PGD_3 after UVR, fatty acid treated and irradiated cells were compared to irradiated cells (FA(-)/UVR (+)) and this results are shown in Figure 6.17B. We observed that low dose of EPA (10 µM) had no significantly effect on PGE_3 and PGD_3 when compared to irradiated cells. However, a significantly increase in PGE_3 was observed when 46BR.1N cells were treated with 50µM EPA ($p \leq 0.001$) and were exposed to 15mJ/cm^2 UVR comparing to the irradiated corresponding control (FA(-) / UVR (+)) (Figure 6.17 and 6.18). This was not noted for PGD_3.

Finally, when cells were treated with DHA (10 and 50 µM) PGE_3 and PGD_3 appeared increased. Also there were no significant differences on PGE_3 and PGD_3 levels when the 46BR.1N cells were treated with 10 and 50 µM DHA and exposed to 15 mJ/cm^2. (Figure 6.17C and 6.18C). .

A summary of the effect of UVR and n-3 PUFA on series-1, -2 and -3 prostanoids are shown in Table (6.3).

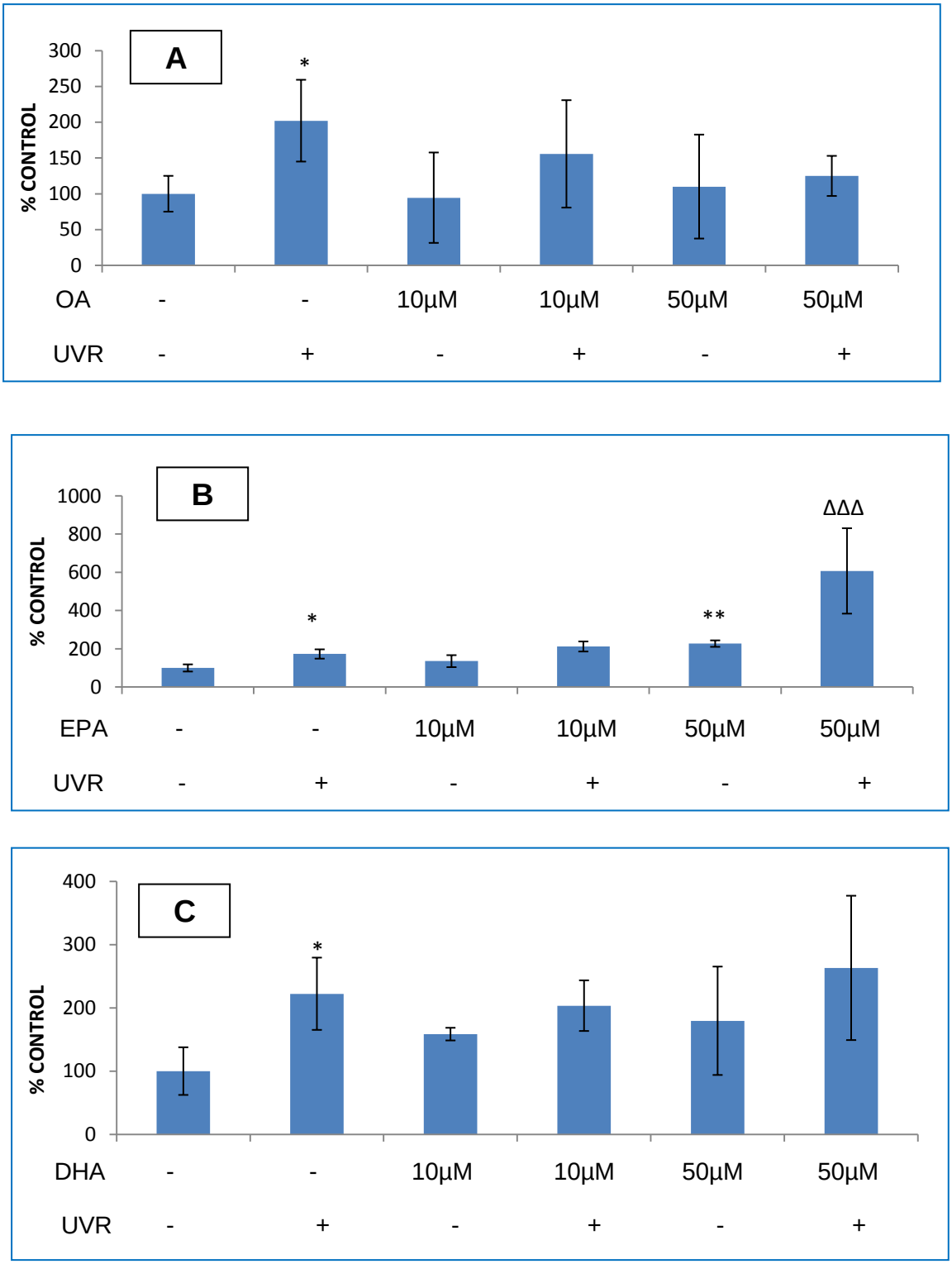


Figure 6.17. Effect of UVR and fatty acid treatment, (A) oleic acid (OA), (B) eicosa pentaenoic acid (EPA) and (C) docosahexaenoic acid (DHA) on PGE_3 produced by 46BR.1N fibroblasts 24 h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *≤0.05, **≤0.01 ΔΔΔp≤0.001, *=comparing to non-irradiated control (FA(-)/UVR(-)). Δ=comparing to irradiated control (FA (-)/UVR(+)).

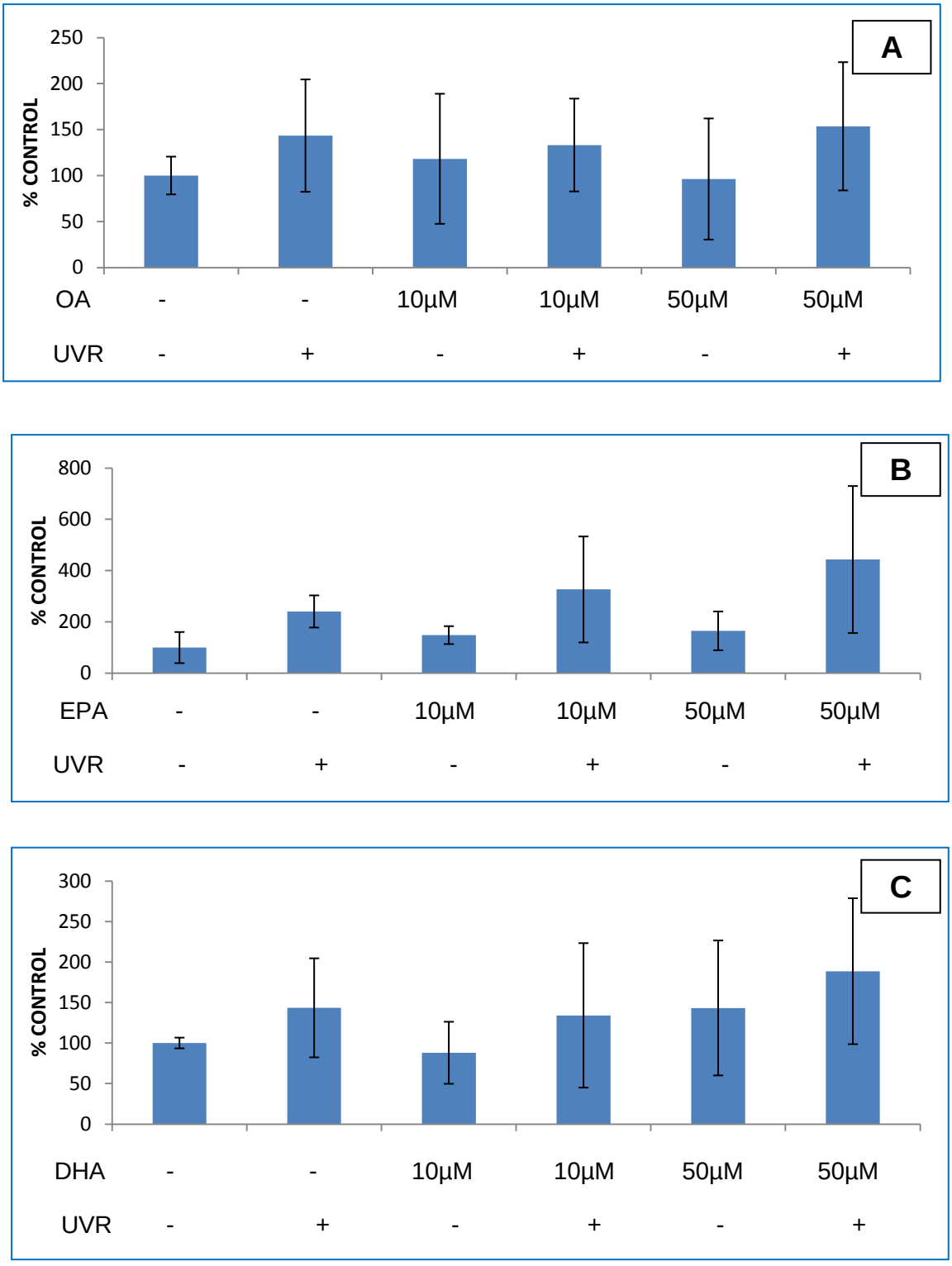


Figure 6.18. Effect of UVR and fatty acid treatment, (A) oleic acid (OA), (B) eicosapentaenoic acid (EPA) and (C) docosahexaenoic acid (DHA) on PGD_3 produced by 46BR.1N fibroblasts 24 h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate).

Table 6.3. Summary of the effect of UVR and n-3 PUFA treatment on the prostanoid mediators produced by 46BR.1N fibroblasts

	UVR (15mJ/cm^2)	No UVR		UVR (15mJ/cm^2)	
Compound		**EPA**	**DHA**	**EPA**	**DHA**
PGE_1	↑*	↓*	—	↓Δ	↓Δ
PGD_1	—	—	—	—	—
PGE_2	↑*	—	—	↓Δ	↓Δ
PGD_2	—	—	—	—	—
PGE_3	↑*	↑*	—	↑Δ	—
PGD_3	—	—	—	—	—
15-keto PGE_2	↑*	—	—	—	—
13,14dihydro-15-PGE_1	↑*	—	—	—	—
13,14dihydro-15-PGE_2	—	—	—	—	—

—= No significant effect

↓= Significant decrease

↑= Significant increase

*= compared to untreated cells (FA(-)/UVR (-))

Δ= compared to untreated irradiated cells (FA(-)/UVR (+))

6.4. Discussion

This study has demonstrated that prostaglandin profiles of both HaCaT keratinocytes and 46BR.1N fibroblasts cells were the same. Moreover, the results show that 46BR.1N fibroblasts produced more prostaglandins than HaCaT keratinocytes as shown in Table 6.1. The results also show that the concentration of PGE_2 and its inactivation products were great than PGE_1 and PGE_3 and their inactivation products. This may be because the level of AA, which is the precursor of PGE_2, in these cells is more than DGLA and EPA, which are the precursors of PGE_1 and PGE_3 respectively. This is supported by the analysis of the contents of these fatty acids in these cells, the data on which are shown in Table 5.1. The results of this study shown that PGE_2 is the main PG in HaCaT at 12.17% of total lipid and at 17.6% in 46BR.1N fibroblasts. It has been reported that PGE_2 (26%) was the major PGs product in cultured human skin fibroblasts (Mayer et al., 1984), and the results of this study agree with this.

It has been reported that UVR is responsible for cutaneous damage after acute or chronic exposure. This causes many cellular and pathological changes including DNA damage and release of pro-inflammatory mediators such as PGE_2 (Filip et al., 2011). Use of photoprotective agents is one way of protecting skin from the harmful effects of UVR. Recently, natural compounds have been considered as skin protectors. In this study, we have shown that n-3 PUFA could be an effective therapeutic natural material for protection against UVB-induced inflammatory mediators.

The results of this study show that, when HaCaT cells were exposed to 15 mJ/cm^2 UVR, the concentration of PGE_1 and PGE_2 were significantly

increased (Figures 6.1 and 6.4). This is in agreement with other reports showing that an increased PGE_2 level was found in HaCaT cells exposed to 6 mJ/cm^2 UVR (Judson et al., 2010). It has been reported that when HaCaT cells were exposed to 60 mJ/cm^2 UVB, the level of PGE_2 also increased compared to non-irradiated cells. Another study reported increases in PGE_2 levels when HaCaT cells were exposed to100mJ/cm^2, and after 48h (Storey et al., 2007). PGE_2 concentration was also increased when HaCaT cells were exposed to 250J/m^2 (Chen et al., 2001). Buckman et al (1998) found increased PGE_2 when primary adult human keratinocytes were irradiated with 30 mJ/cm^2 UVB (Buckman et al., 1998). Increase in PGE_2 was also observed in normal human keratinocytes when were exposed to 32 mJ/cm^2 UVB (Pupe et al., 2002). Furthermore, in a clinical study, the level of PGE_2 was increased in association with increased COX-2 expression in 32 healthy volunteers exposed to 4-MED for 72 h (Rhodes et al., 2009).

It is possible that UVR increased levels of PGs found in skin may also reflect the reduced catabolism of these PGs in addition to an increase in their synthesis. This could be attributed to increased expression of COX-2, which plays a major role in PG synthesis, and a decrease or suppression of the expression of 15-PGDH, which plays a major role in PG catabolism. Findings in the present study suggest that both PGE_1 and PGE_2 and their inactivation products, 13,14-dihydro-15-keto-PGE_1, 13,14-dihydro-15-keto-PGE_2 and 15-keto-PGE_2, were increased post UVR treatment. Increased levels of the inactivation products of PGE_1 and PGE_2 may be a normal result of the cell haemostasis. Increased levels of PG inactivation were reported by Rhodes et al (2009). They reported that 13,14-dihydro-15-keto-PGE_1, 13,14-dihydro-15-keto-

PGE_2 and 15-keto-PGE_2 were significantly increased post UVR in 32 healthy humans. However, Judson et al reported that UVR (6 mJ/cm^2 UVB) decreased 15-PGDH protein, mRNA and enzyme activity in HaCaT cells. (Judson et al., 2010). We also found that PGE_3 concentration was increased after UVR treatment, as also reported by Rhodes et al (2009).

The results suggest that treating HaCaT cells with OA had no effect in the production of COX mediators. According to the hypothesis and experimental design, this was expected. Furthermore, these results confirm that OA, which was chosen as a control in this study did not convert into EPA or DHA. Conversely, n-3 PUFA EPA and DHA increased PGE_3 and decreased PGE_1 and PGE_2 post 15 mJ/cm^2 UVR. Other studies are in agreement with our results; normal human keratinocyte treated with 50 μM EPA resulted in a marked decrease in PGE_2 level post 32 mJ/cm^2 UVB, which was most apparent at 24h post irradiation, and PGE_2 levels were reduced four-fold compared to non-irradiated cells (Pupe et al., 2002). In a study where HaCaT cells were treated with 50μM EPA and DHA this was found to inhibit UVB-induced inflammation by modulation the interleukin (IL)-8 but there was no such effect when the cells were treated with 50μM OA (Storey et al., 2007). In contrast, induced COX-2 expression through increased activation of γPPAR was found when HaCaT cells were treated with DHA (Chene et al., 2007)

The results of this study show that treating 46BR.1N cell fibroblasts with OA, EPA and DHA had no effect on the baseline level of series-1 prostaglandins (PGE_1, PGD_1 and 13,14-dihydro-15-ketoPGE_1). Although the levels of these prostaglandins increased when 46BR.1N cell were exposed to 15mJ/cm^2, this was not affected by the OA, EPA and DHA treatment. Our

results also showed that there was no effect of these fatty acids on series-2 prostaglandins at baseline (Figure 6.13-16). Exposing the cells to 15mJ/cm^2 showed statistically significant changes in the levels of PGE_2. A similar increase in PGE_2 level was observed when CCD922SK normal human breast skin fibroblasts were exposed to 100mJ/cm^2 UVB (Storey et al., 2007), and when cultured human skin fibroblasts were treated with 150mJ/cm^2 UVB (Filipe et al., 1995).

Treatment of 46BR.1N with OA did not affect the UVB-induced PGE_2 production. EPA reduced the level of PGE_2 but this was not statistically significant and only DHA had a significant effect and reduced the UVB-induced PGE_2. Treating CCD922SK cells (skin fibroblasts) with 50μM EPA and DHA inhibited UVB induced inflammation by modulation of IL-8 (Storey et al., 2007).

Chapter 7. Effect of UVR and n-3 PUFA on hydroxy fatty acids production in skin cells

7.1 Introduction

Lipoxygenases found in skin can metabolise C18 and C20 PUFA to produce predominantly monohydroxy fatty acids: AA is metabolised by 5-LOX to 5-HETE, by 12-LOX to 12-HETE and by 15-LOX to 15-HETE (Ziboh, 1992). It has been suggested that 5-HETE itself does not play a significant role in biological signalling, but it is further reduced by 5-hydroxyeicosatetraenoic acid dehydrogenase to form the bioactive 5-oxo-eicosatetraenoic acid (5-oxoETE) (Powell and Rokach, 2005). Studies in human neutrophil reported that 5-oxoETE is a potent stimulator of calcium mobilisation and cell migration. 5-HETE has a similar role to 5-oxoETE but it is as much as 100 times less (Powell et al., 1993). An *in vivo* study has shown that intradermal injection of 5-oxoETE in humans led to infiltration of neutrophils into the skin (Muro et al., 2003). Moreover, 5-oxoETE has been found to be a potent simulator of human eosinophil migration with a potency up to 30 times greater than LTB_4 (Powell et al., 1995). These results suggest that 5-oxo-ETE may be an important mediator of inflammation.

12-HETE is the main AA metabolite found in skin (Ruzicka et al., 1983), and is believed to have both a physiologic role in the biology of cutaneous reparative processes and pathophysiologic effects in inflammatory skin diseases such as psoriasis, atopic eczema and contact dermatitis (Black et al., 1985a, Dowd et al., 1987). intradermal injection of 12-HETE in guinea pigs led to an increase in the number of infiltrated neutrophils and eosinophils into the skin (Waldman et al., 1989). It has also been reported that 12-HETE can be a chemo-attractant for lymphocytes in addition to neutrophils (Bacon et al., 1988).

15-HETE and 15-HETrE are believed to have potent anti-inflammatory properties (Ziboh, 1992). The supplementation of diets with appropriate purified vegetable oil and/or fish oil may help to improve cutaneous inflammatory reactions and disorders (Ziboh, 1992). Many studies found that supplementation with DGLA (precursor of 15-HETrE) led to improvement in atopic dermatitis (Wright and Burton, 1982, Fiocchi et al., 1994) and eczema (Henz et al., 1999). It has been reported that LTB_4 induced skin reaction which lasted up to 18 h after the injection. A significant decrease was observed when LTB_4 was injected together with 300 ng of 15-HETE and the skin reaction was reduced 1 h after the injection. These results support the role of 15-HETE as a pro-inflammatory mediator (Ternowitz et al., 1989). Furthermore, dermis-derived 15-HETE inhibits epidermal 12-LOX activity (Kragballe et al., 1986), and significant inhibition of 12-LOX expression was found when HaCaT cells were treated with 15-HETE (Yoo et al., 2008).

The lipid mediator system in the body depends on the presence of PUFA and the ratio of n-6 PUFA to n-3 PUFA. Supplementation with n-3 PUFA has been shown to increase the level of anti-inflammatory mediators (Yang et al., 2004, Bagga et al., 2003). With the use of mass spectrometry, it has been shown that EPA and DHA generate potent anti-inflammatory mediators that are implicated in the resolution of inflammation. 18-HEPE and 17-HDHA have been identified as precursors of RvE and RvD respectively (Hong et al., 2003, Marcheselli et al., 2003, Arita et al., 2005). However, this has not yet been shown in the skin.

UVR is one of main factors capable of modulating various cell-surface molecules. It has been reported that treating HaCaT cells with 100, 200 and

300J/m^2 UVB has produced a significant dose-dependent decrease of 12-LOX and a significant dose-dependent increase of 15-LOX expression (Yoo et al., 2008). The same result was also found when HaCaT cells were exposed to 10, 20 and 30 J/m^2 UVA (Yoo et al., 2008). A study of 32 healthy adult subjects showed up-regulation of 12-HETE and 15-HETE production in human skin expression following UVR exposure (Nicolaou et al., 2012, Rhodes et al., 2009). Furthermore, exposure of the human epidermal cell line SCL-II to 100-300 J/m^2 UVB led to the modulation of 12-HETE receptors and a large decrease in 12-HETE binding sites (Kemeny et al., 1991).

This part of the study aimed to study the hydroxy fatty acid production by HaCaT keratinocytes and 46BR.1N fibroblasts, and anwser the following question:

A) What kind of hydroxy fatty acids are produced by these cells?
B) Is there any effect of UVR in hydroxy fatty acids production?
C) What is the effect of n-3PUFA in hydroxy fatty acid production with and without UVR treatment?

7.2. Methods

All information relevant to the experiments reported in this study is presented in section 2.2. Cell culture and passaging in section 2.2.3.2 and cell counting in section 2.2.3.2. Exposure of cells to UVR is in section 2.2.3.3.2. Section 2.2.3.4 describes the treatment of cells with fatty acids and section 2.2.3.5 shows the collection of cells and media. Hydroxy fatty acid analysis is reported in section 2.4 including sample preparation and LC/ESI-MS/MS analysis.

7.3 Results

7.3.1. Hydroxy fatty acid profile in HaCaT and 46BR.1N cells

In order to measure the baseline concentration of hydroxy fatty acids produced by HaCaT keratinocytes and 46BR.1N fibroblasts, the cells were cultured and medium was collected and analysed by LC/ESI-MS/MS. 25 Hydroxy fatty acids were detected in both cells (Table 7.1).

Table 7.1 shows the concentration (pg/million cell) of the hydroxy fatty acids present in HaCaT keratinocytes and 46BR.1N fibroblasts. It is clear that 46BR.1N produce more hydroxy fatty acids than HaCaT cells. HODE are formed from LA. The concentration of 13-HODE was high than 9-HODE and this was found to be the most abundant hydroxy fatty acid in HaCaT (15.7%) and 46BR.1N (28.3%) cells. EPA is the precursor of HEPE. Seven types of HEPE were found in both cells with 15-HEPE being the main HEPE followed by 9-HEPE. 15-HEPE was the third abundant hydroxy fatty acid in HaCaT (9.3%) and 46BR.1N cells (8%). By the action of lipoxygenases AA is converted to HETE. 12-HETE which is generated by the action of 12-LOX was found at higher concentration in HaCaT (12%) and 46BR.1N (11%). 15-HETE which is generated by 15-LOX was found at lower concentration on 3.3% and 3.1%, respectively. DHA is the precursor of the HDHA. 17-HDHA is generated by the action of 15-LOX and was found in both HaCaT (7.3%) and 46BR.1N (4.9%).

Table 7.1. Profile of hydroxy fatty acids produced by HaCaT and 46BR.1N.

	HaCaT	HaCaT	46BR.1N	46BR.1N
	pg/million cell	% of total	pg/million cell	% of total
9-HODE	10.78 ± 5.0	9.1	33.7 ± 11.7	5.4
13-HODE	18.52 ± 6.8	15.7	188.1 ± 18.7	28.3
5-HEPE	1.47 ± 0.1	1.2	2.9 ± 1.0	0.4
18-HEPE	2.32 ± 1.3	2.0	20.0 ± 1.7	3.0
9-HEPE	3.83 ± 2.1	3.3	50.4 ± 24.4	7.6
8-HEPE	3.83 ± 1.9	3.3	3.8 ± 0.9	0.6
11-HEPE	3.19 ± 0.6	2.7	34.7 ± 15.2	5.2
15-HEPE	11.00 ± 2.7	9.3	52.9 ± 14.9	8.0
12-HEPE	1.79 ± 0.6	1.5	8.1 ± 2.4	1.2
5-OXOETE	3.98 ± 1.6	3.4	4.2 ± 3.8	0.6
14(15)EET	2.51 ± 0.9	2.1	4.2 ± 3.8	0.6
5-HETE	2.45 ± 0.6	2.1	6.1 ±0.3	0.9
9-HETE	2.88 ± 1.0	2.4	2.5 ± 0.7	0.4
8-HETE	1.78 ± 0.7	1.5	3.3 ± 1.2	0.5
11-HETE	1.12 ± 0.3	1.0	24.0 ± 5.1	3.6
15-HETE	3.94 ± 1.0	3.3	20.5 ± 4.4	3.1
12-HETE	13.57 ± 8.6	11.5	73.4 ± 9.6	11.0
15-HETrE	0.65 ± 0.2	0.5	8.2 ± 1.9	1.2
14,15-DHET	1.78 ± 0.5	1.5	3.9 ± 2.0	0.6
10-HDHA	2.39 ± 1.6	2.0	2.7 ± 0.6	0.4
14-HDHA	5.33 ± 3.5	4.5	10.3 ± 4.4	1.6
13-HDHA	1.79 ± 1.4	1.5	23.1 ± 3.1	3.5
17-HDHA	8.37 ± 2.3	7.1	32.6 ± 10.6	4.9
20-HDHA	8.65 ± 3.2	7.3	8.5 ± 1.3	1.3

7.3.2. The effect of fatty acid and UVR treatment on hydroxy fatty acids produced by HaCaT keratinocytes

7.3.2.1. The effect of fatty acid and UVR treatment on HODE

As shown in Figure 7.1 the levels of 9-HODE and 13-HODE appeared increased when HaCaT cells were exposed to 15mJ/cm^2 UVR. However, this was statistically significantly ($p \leq 0.05$ and $p \leq 0.05$, respectively) when compared to non-irradiated control (FA(-)/UVR(-)).

In order to assess the effect of FA treatment on HODE production by HaCaT cells, cells were treated with 10 and 50μM of OA, EPA and DHA and exposed to UVR. Media were collected and analysed. HaCaT cells treated with OA 10 and 50μM showed no significant effect on 9-, 13-HODE at baseline or post UVR when compared to corresponding control (Figures 7.1).

HaCaT treated with 10 and 50μM EPA showed no significant decreased on 9-HODE and 13-HODE at baseline compared to non-irradiated control (FA(-)/UVR(-)). Significant decrease in 9-,13-HODE ($p \leq 0.01$, $p \leq 0.05$, respectively) levels was when HaCaT cells were treated with 50μM EPA and exposed to 15mJ/cm^2 compared to irradiated control (FA (-)/UVR(+)).(Figures7.1)

Finally, HaCaT cells treated with 10 and 50μM DHA showed no significant decreased on 9-,13-HODE at the baseline compared to non-irradiated control (FA(-)/UVR(-)). Significant decrease in 9-HODE ($p \leq 0.05$, $p \leq 0.01$) level was observed when the cells were treated with 10 and 50μM DHA, respectively. While 13-HODE ($p \leq 0.05$) was significant decrease when the cells were treated with 50μM DHA compared to irradiated control (FA (-)/UVR(+)). Results are shown in Figures 7.1.

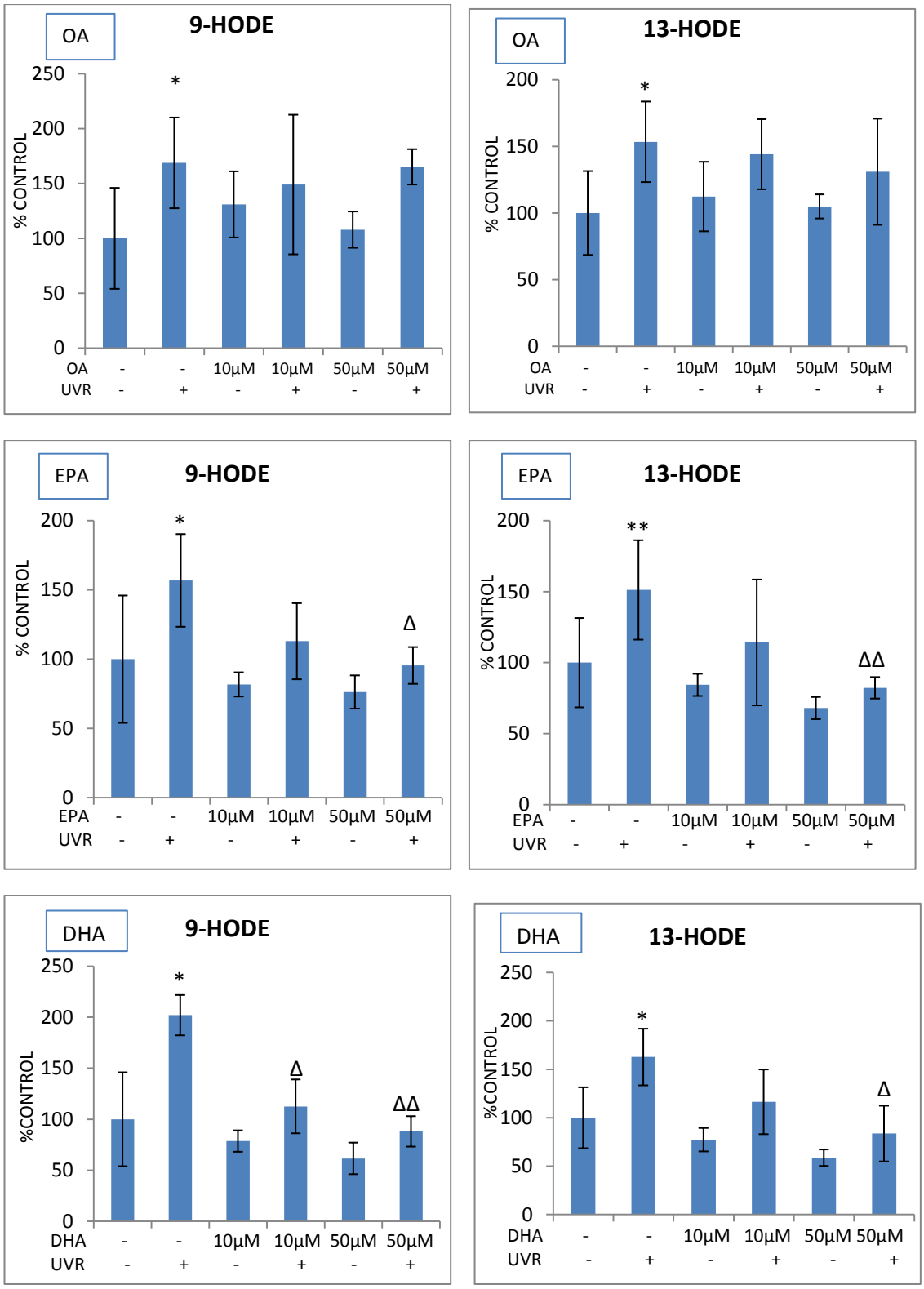


Figure 7.1. Effect of UVR and fatty acid treatment, oleic acid (OA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on HODE produced by HaCaT keratinocyte 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10μM and 50 μM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *Δp≤0.05, **ΔΔp≤0.01, *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

7.3.2.2. The effect of UVR and fatty acid treatment on HEPE

Seven HEPE were detected in HaCaT. However, 5 mediators were significantly increased 5-,9-,11-,12- and 18-HEPE ($p \leq 0.05$, $p \leq 0.01$,$p \leq 0.001$, $p \leq 0.05$ and $p \leq 0.01$; respectively) when cells were exposure to 15mJ/cm^2 UVR, compared to non-irradiated control (FA(-)/UVR(-)).Figure (7.2-4).

As shown in Figure 7.2, there was no effect of OA (10 and 50 µM) on the level of HEPE mediators at the baseline when compared to non-irradiated control (FA(-)/UVR(-)). Also no significant difference on HEPE mediators was found when 10, 50 µM OA treated HaCaT were exposed to 15mJ/cm^2 compared to irradiated control (FA (-)/UVR(+)).

Treated HaCaT cells with EPA (10 and 50 µM) showed significant increased on the level of 5-, 9-, 12-, 15- and 18-HEPE (($p \leq 0.05$,0.01), ($p \leq 0.01$,0.01), ($p \leq 0.05$,0.05), ($p \leq 0.05$,0.05) and ($p \leq 0.05$, 0.05), respectively), when compared to non-irradiated control (FA(-)/UVR(-)). An increase in these mediators was also observed when HaCaT cells treated with 10 and 50 µM of EPA were exposed to 15 mJ/cm^2 UVR, but only 5-,15-HEPE were statistically significant ($p \leq 0.05$ and 0.005) after 50 µM of EPA. (Figure 7.3)

HaCaT treated with 10 µM of DHA showed no significant increase in HEPE mediators. However, treated with 50 µM DHA showed significant increase in 5-,9-,11-,12-,15- and 18-HEPE ($p \leq 0.01$, $p \leq 0.01$, $p = 0.05$, $p \leq 0.05$, $p \leq 0.05$ and $p \leq 0.01$; respectively) compared to non-irradiated control (FA(-)/UVR(-)). Also, 8-,12-HEPE were significant increase ($p \leq 0.01$ and $p \leq 0.05$; respectively) when HaCaT treated with 50 µM of DHA exposed to 15mJ/cm^2 UVR and compared to irradiated control (FA (-)/UVR(+)). (Figure 7.4).

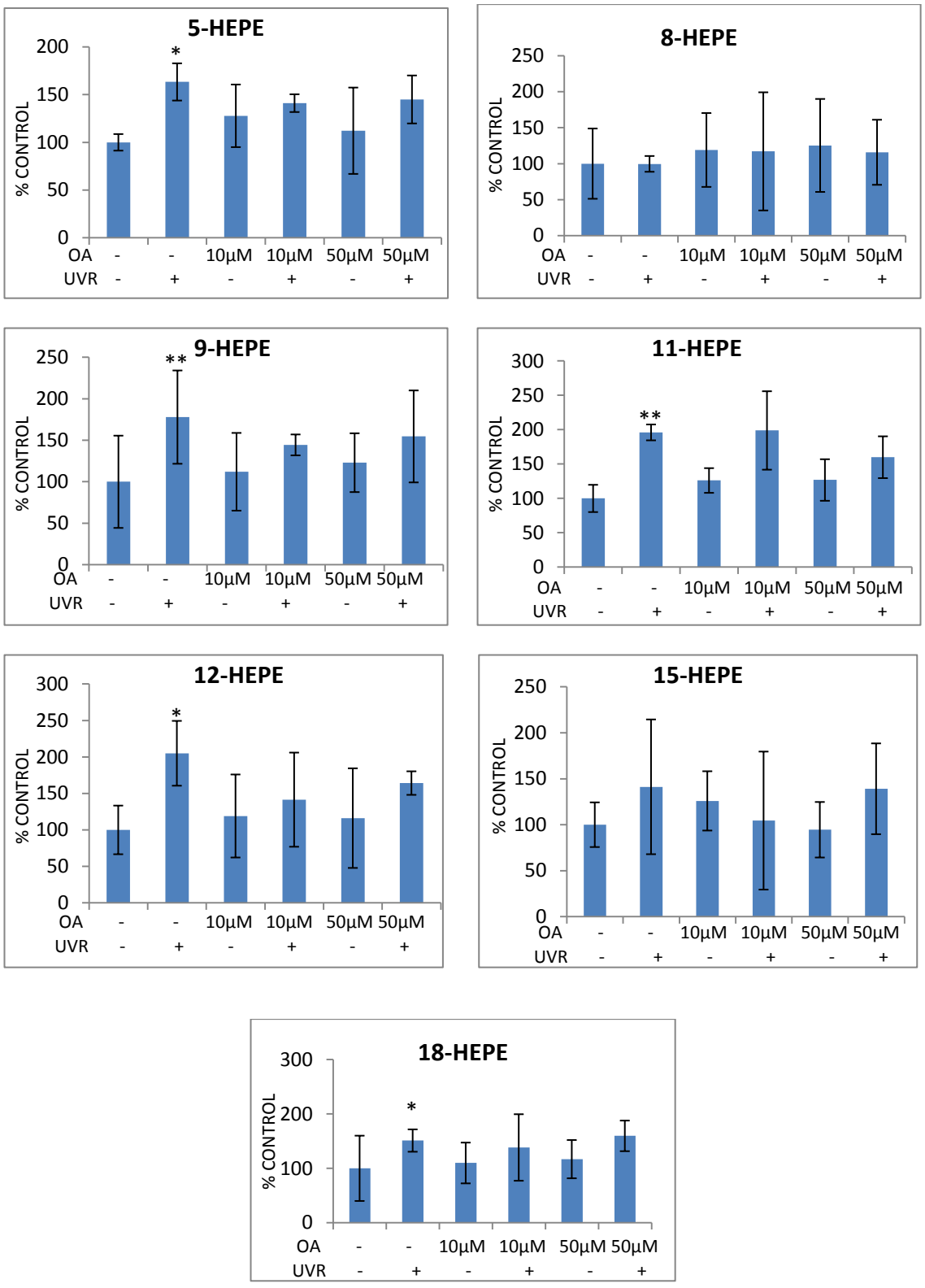


Figure 7.2. Effect of UVR and oleic acid (OA) treatment on hydroxy-eicosapentaenoic acid (HEPE) mediators produced by HaCaT keratinocytes 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10μM and 50 μM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *p≤0.05, **p≤0.01, *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

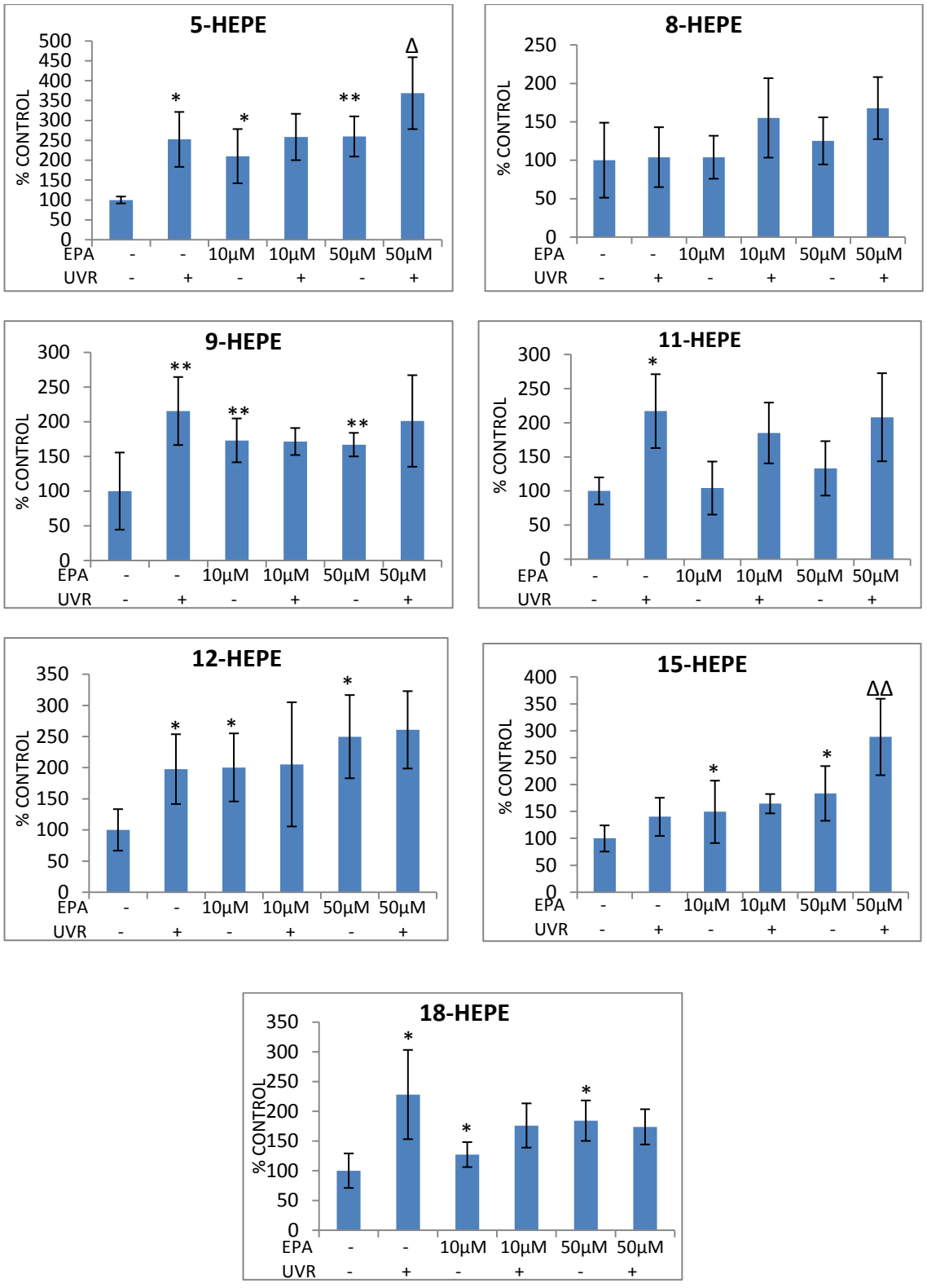


Figure 7.3. Effect of UVR and eicosapentaenoic acid (EPA) treatment on hydroxy-eicosapentaenoic acid (HEPE) mediators produced by HaCaT keratinocytes 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10μM and 50 μM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *p≤0.05, **,ΔΔp≤0.01 *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

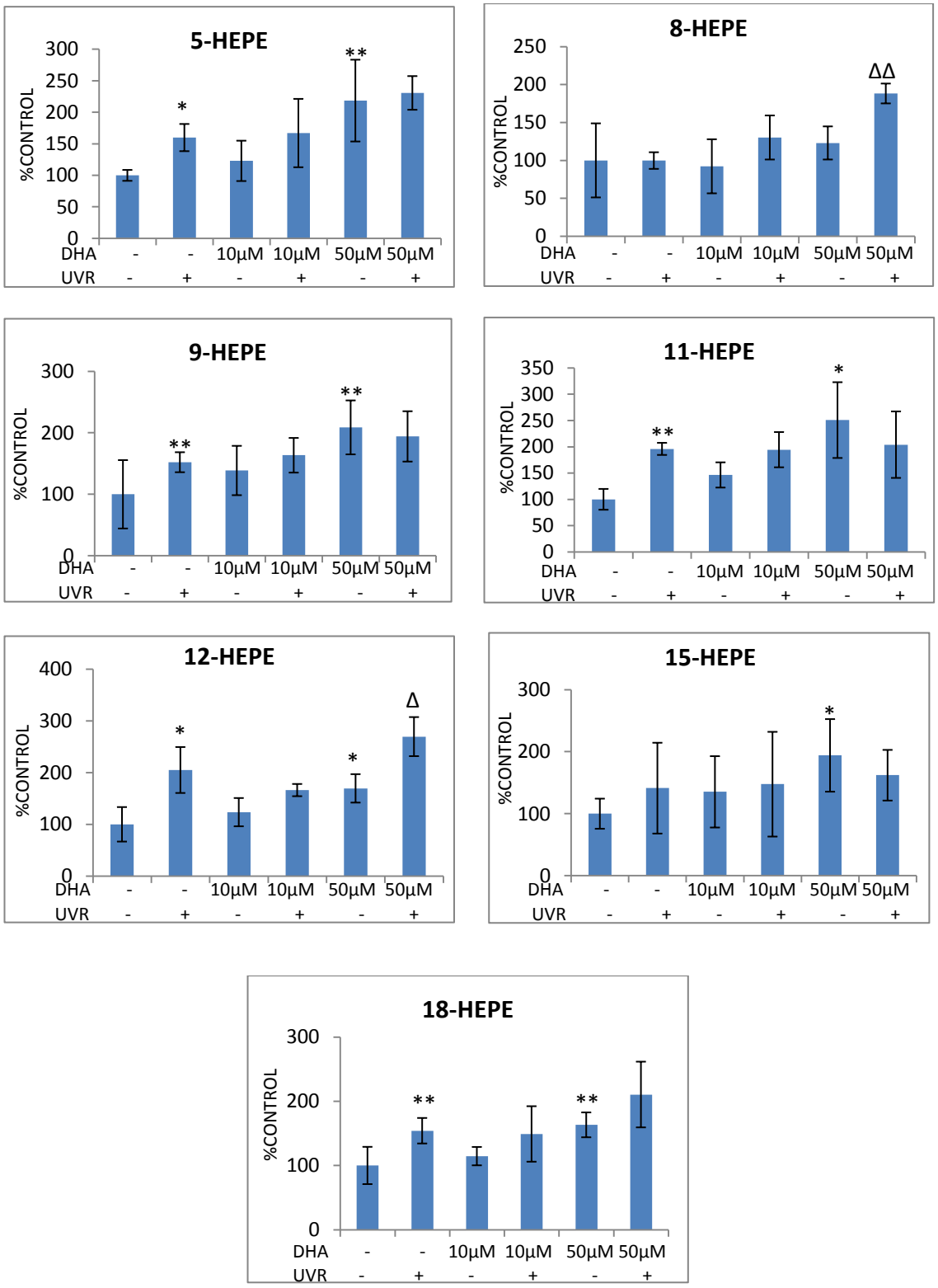


Figure 7.4. Effect of UVR and docosahexaenoic acid (DHA) treatment on hydroxy-eicosapentaenoic acid (HEPE) mediators produced by HaCaT keratinocytes 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10μM and 50 μM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *p≤0.05, **,ΔΔp≤0.01 *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

7.3.2.3. The effect of UVR and fatty acid treatment on HETE

Six HETE were detected in HaCaT keratinocytes. Exposure to15mJ/cm^2 UVR shown significant increase in the level of 5-HETE ($p \leq 0.05$), 8-HETE ($p \leq 0.05$), 9-HETE ($p \leq 0.05$), 11-HETE ($p \leq 0.01$), 12-HETE ($p \leq 0.01$) and 15-HETE ($p \leq 0.01$) when compared to non-irradiated control (FA(-)/UVR(-)).

Figure 7.5 shows the effect of OA (10 and 50 µM) on the level of HETE at baseline, there was no significant difference when compared to non-irradiated control (FA(-)/UVR(-)). Also no significant difference on HETE was found when OA (10 and 50 µM) treated HaCaT were exposed to 15mJ/cm^2, compared to irradiated control (FA (-)/UVR(+)).

Although there was a decrease in the level of HETE mediators after treatment with 10 and 50 µM EPA, this was not statistically significant when compared to non-irradiated control (FA(-)/UVR(-)).(Figure 7.6). Significant decreased in 9-HETE ($p \leq 0.05, 0.05$) and 11-HETE ($p \leq 0.05, 0.05$) was observed when HaCaT cells were treated with 10 and 50 µM of EPA and exposed to 15 mJ/cm^2 UVR. Also, significant decreased in the level of 5-HETE ($p \leq 0.05$) and 8-HETE ($p \leq 0.05$) was noted after treatment with 50 µM of EPA. Compared to irradiated control (FA (-)/UVR(+)) (Figure 7.6). No significant decrease in HETE was observed when the cells were treated with 10 and 50 µM of DHA compared to non-irradiated control (FA(-)/UVR(-)). Significant decreased in 5-HETE ($p \leq 0.05, 0.05$), 11-HETE ($p \leq 0.01, 0.05$) and 15-HETE ($p \leq 0.05, 0.01$) was observed when HaCaT cells were treated with 10 and 50 µM of DHA and exposed to 15 mJ/cm^2 UVR. Also, significant decreased in 8-HETE ($p \leq 0.05$) and 9-HETE ($p \leq 0.05$) was observed after treated with 50 µM of DHA. Compared to irradiated control (FA (-)/UVR(+)) (Figure 7.7).

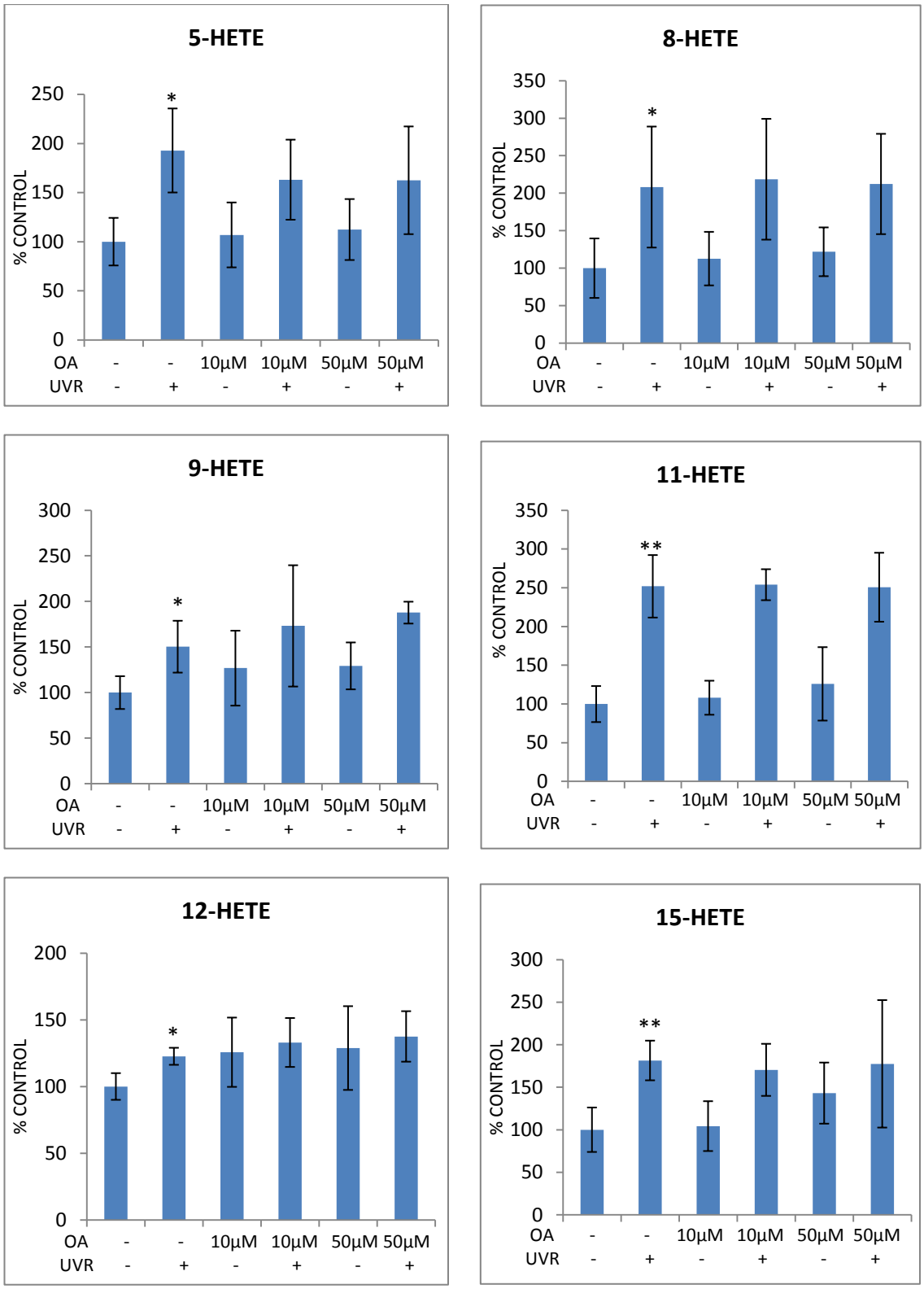


Figure 7.5. Effect of UVR and oleic acid (OA) treatment on Hydroxy-eicosatetraenoic acids (HETE) mediators produced by HaCaT keratinocytes 24h post UVR (15 mJ/cm2). Cells were treated with two concentrations of each fatty acid (10μM and 50 μM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *p≤0.05, **p≤0.01, *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

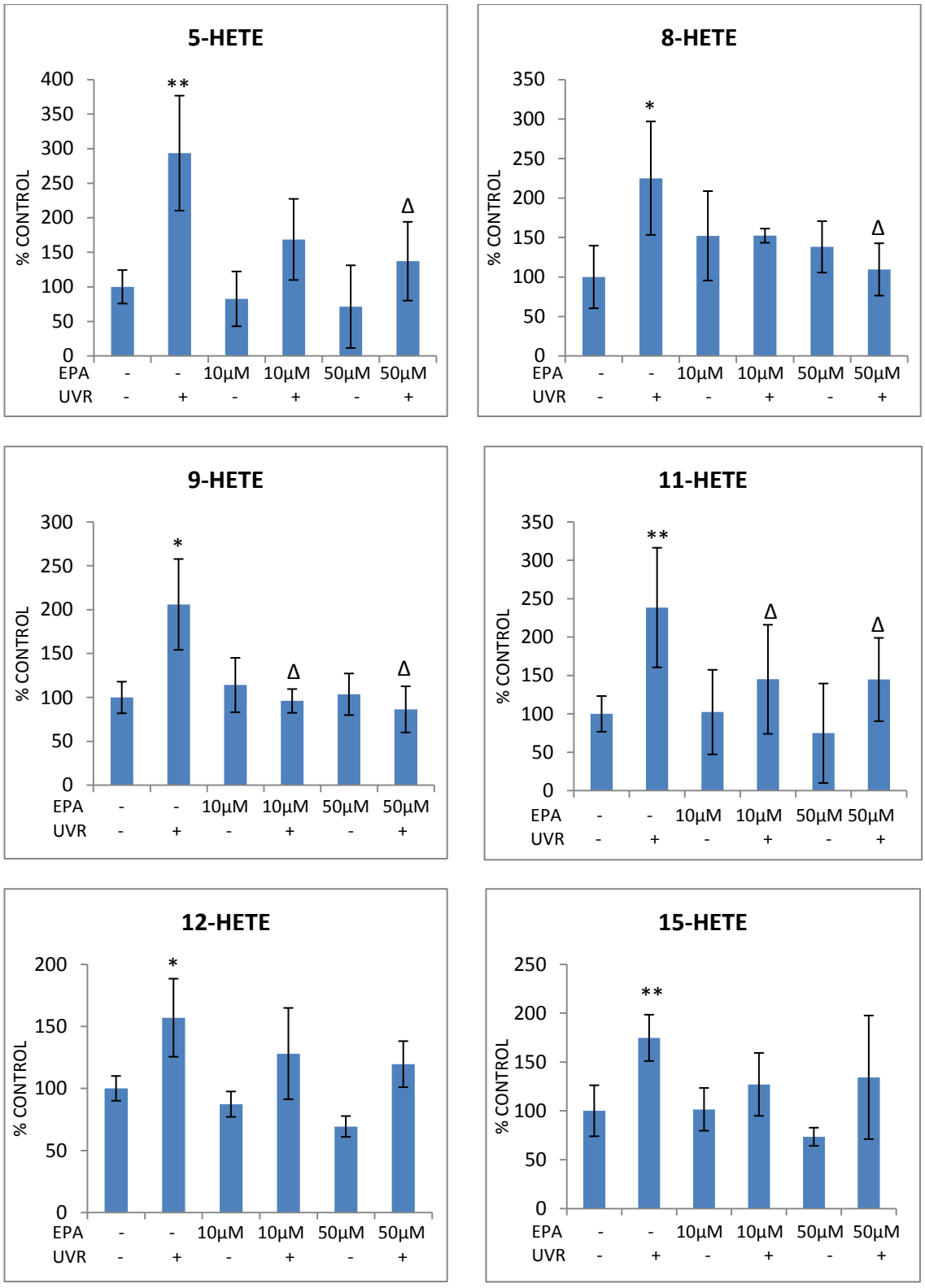


Figure 7.6. Effect of UVR and eicosapentaenoic acid (EPA) treatment on Hydroxy-eicosatetraenoic acids (HETE) mediators produced by HaCaT keratinocytes 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10μM and 50 μM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *p≤0.05, **,ΔΔp≤0.01 *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

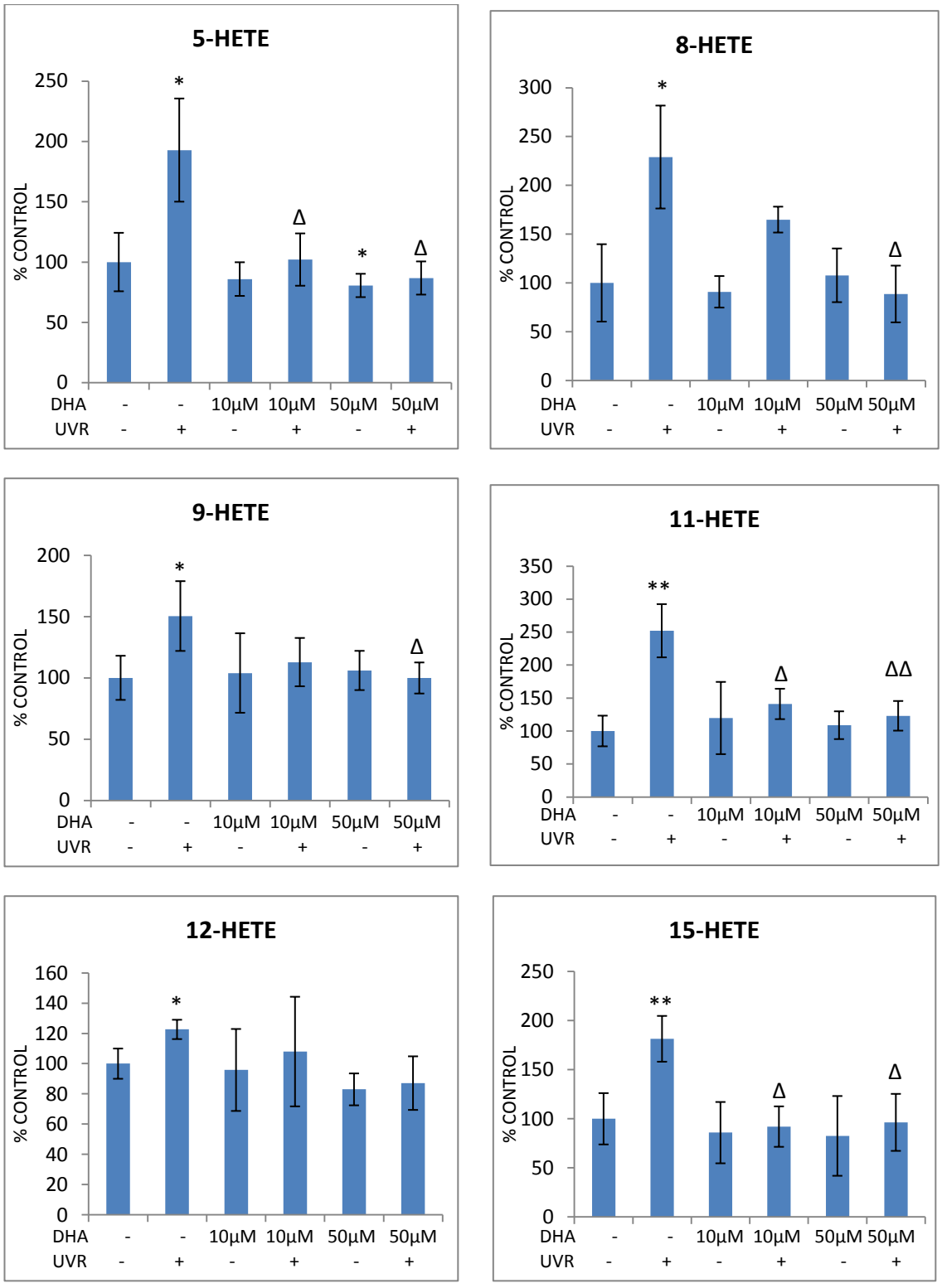


Figure 7.7. Effect of UVR and eicosatetraenoic acid (DHA) treatment on Hydroxy-eicosatetraenoic acids (HETE) mediators produced by HaCaT keratinocytes 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10μM and 50 μM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *p≤0.05, **,ΔΔp≤0.01 *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

7.3.2.4. The effect of UVR and fatty acid treatment on HDHA

Five HDHA were detected in HaCaT cells. Exposing the cells to 15mJ/cm^2 UVR showed significant increased in the level of 13-HDHA ($p \leq 0.05$), 14-HDHA ($p \leq 0.01$), 17-HDHA ($p \leq 0.01$) and 20-HDHA ($p \leq 0.01$) when compared to non-irradiated control (FA(-)/UVR(-)).

Figure 7.8 shows the effect of OA (10 and 50 µM) on the levels of HDHA mediators at the baseline, there was no significant difference when compared to non-irradiated control (FA(-)/UVR(-)). Also no significant difference was found when HaCaT treated with OA (10 and 50 µM) were exposed to 15mJ/cm^2 and the result were compared to irradiated control (FA (-)/UVR(+)).

Statistically significant increase in the level of 13-HDHA ($p \leq 0.001$), 17-HDHA ($p \leq 0.05$) and 20-HDHA ($p \leq 0.01$) was observed when HaCaT cell were treat with 50 µM EPA and compared to non-irradiated control (FA(-)/UVR(-)) (Figure 7.9). Moreover, an increase on these mediators was observed when HaCaT cells were treated with 10 and 50 µM of EPA and exposed to 15 mJ/cm^2 UVR but this was not statistically significant compared to irradiated control (FA (-)/UVR(+)).

Treated HaCaT cell with 10 and 50 µM DHA showed significant increased in 10-, 13-, 14-, 17- and 20-HDHA ($p \leq 0.05$,0.01), ($p \leq 0.05$,0.001), ($p \leq 0.05$, 0.05), ($p \leq 0.05$,0.01) and ($p \leq 0.05$,0.01),respectively, compared to non-irradiated control (FA(-)/UVR(-)). Also, significant increase in 10-HDHA ($p \leq 0.01$),13-HDHA ($p \leq 0.001$),14-HDHA ($p \leq 0.01$) and 17-HDHA ($p \leq 0.01$) was noted when HaCaT cells were treated with 50 µM DHA and exposed to 15mJ/cm^2 compared to non-irradiated control (FA(-)/UVR(-)) (Figure 7.10).

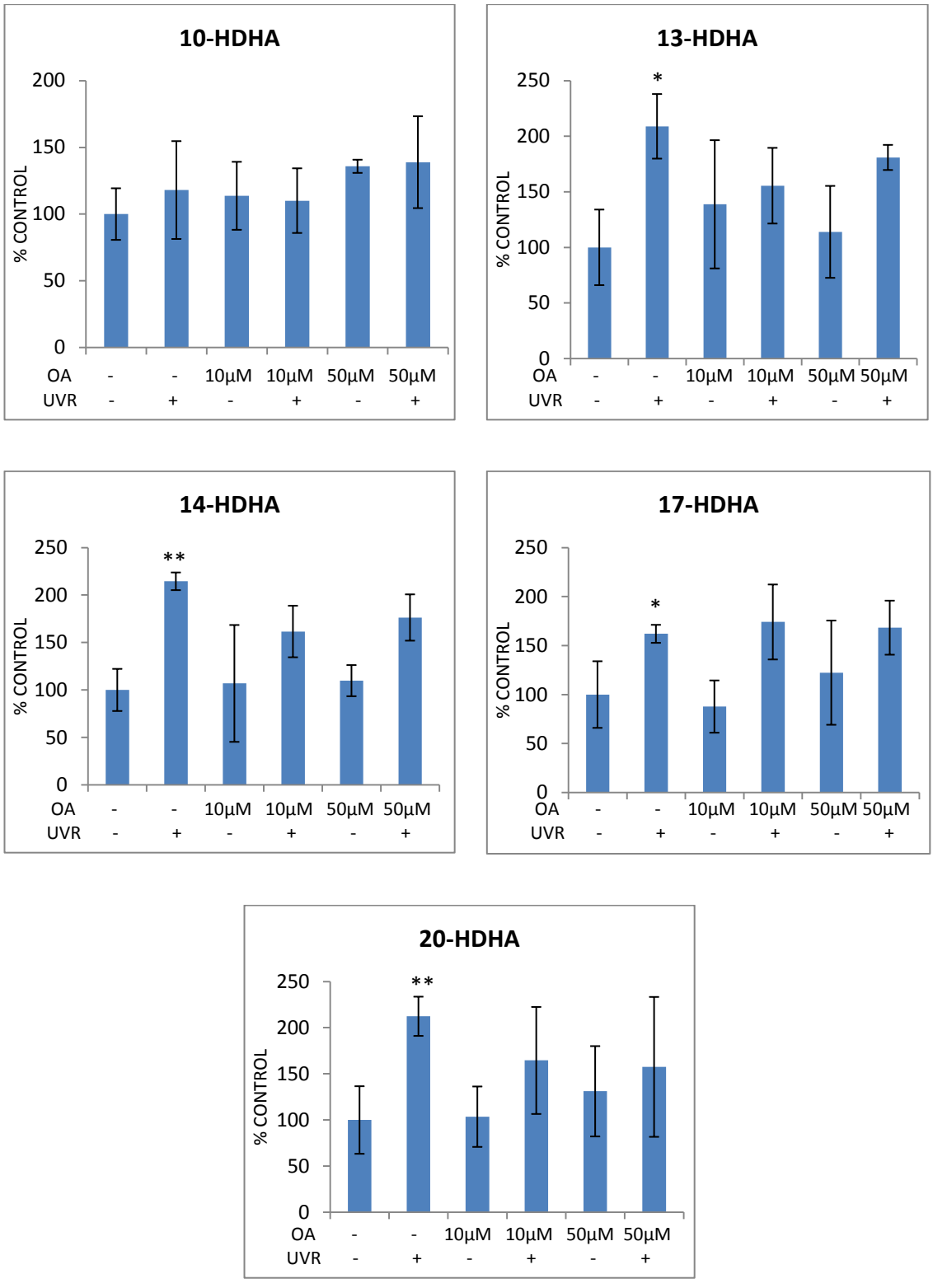


Figure 7.8. Effect of UVR and oleic acid (OA) treatment on hydroxy-docosahexaenoic acid (HDHA) mediators produced by HaCaT keratinocytes 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10μM and 50 μM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *p≤0.05, **p≤0.01, *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

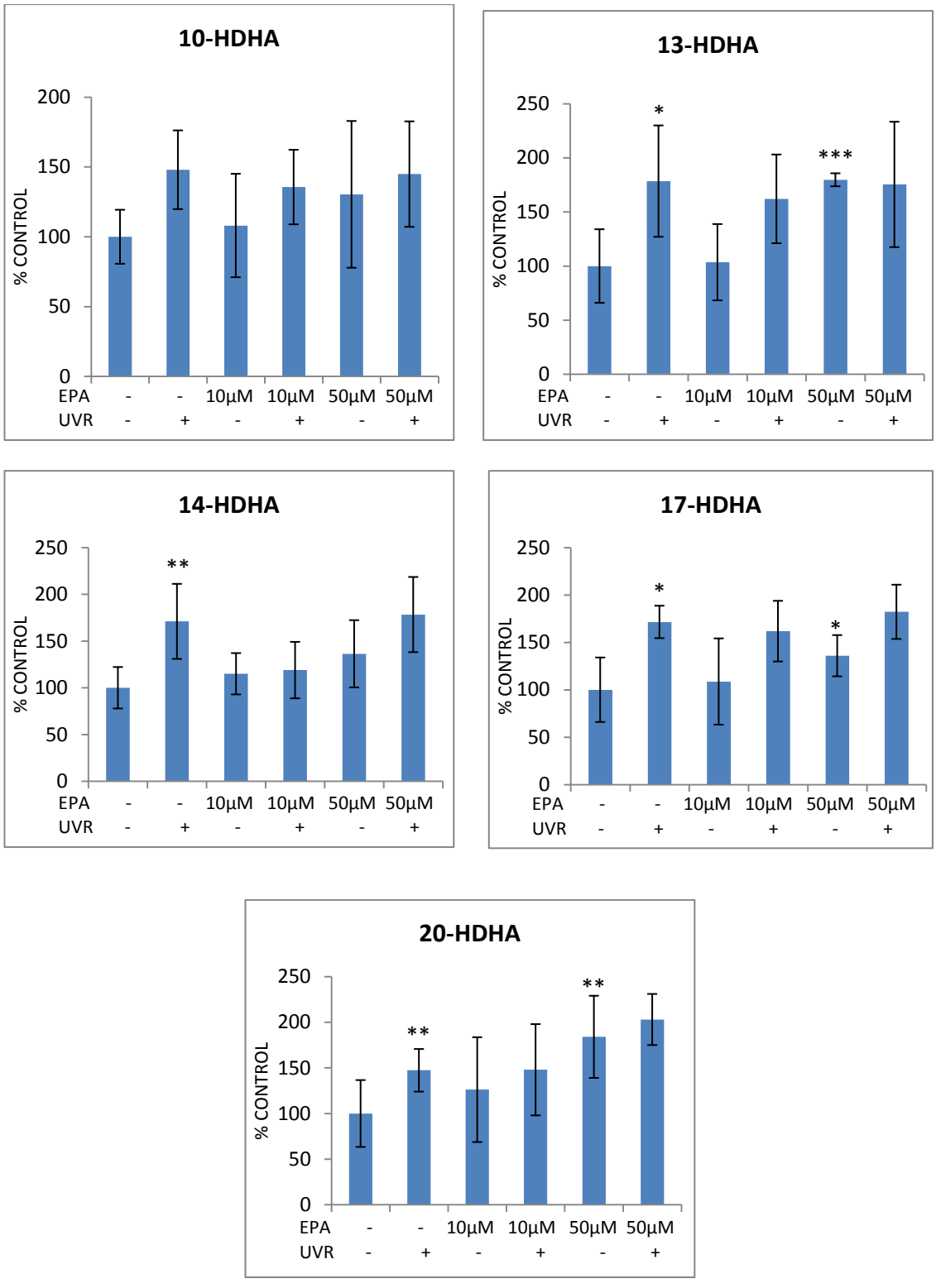


Figure 7.9. Effect of UVR and eicosapentaenoic acid (EPA) treatment on hydroxy-docosahexaenoic acid (HDHA) mediators produced by HaCaT keratinocytes 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *p≤0.05, **p≤0.01, *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

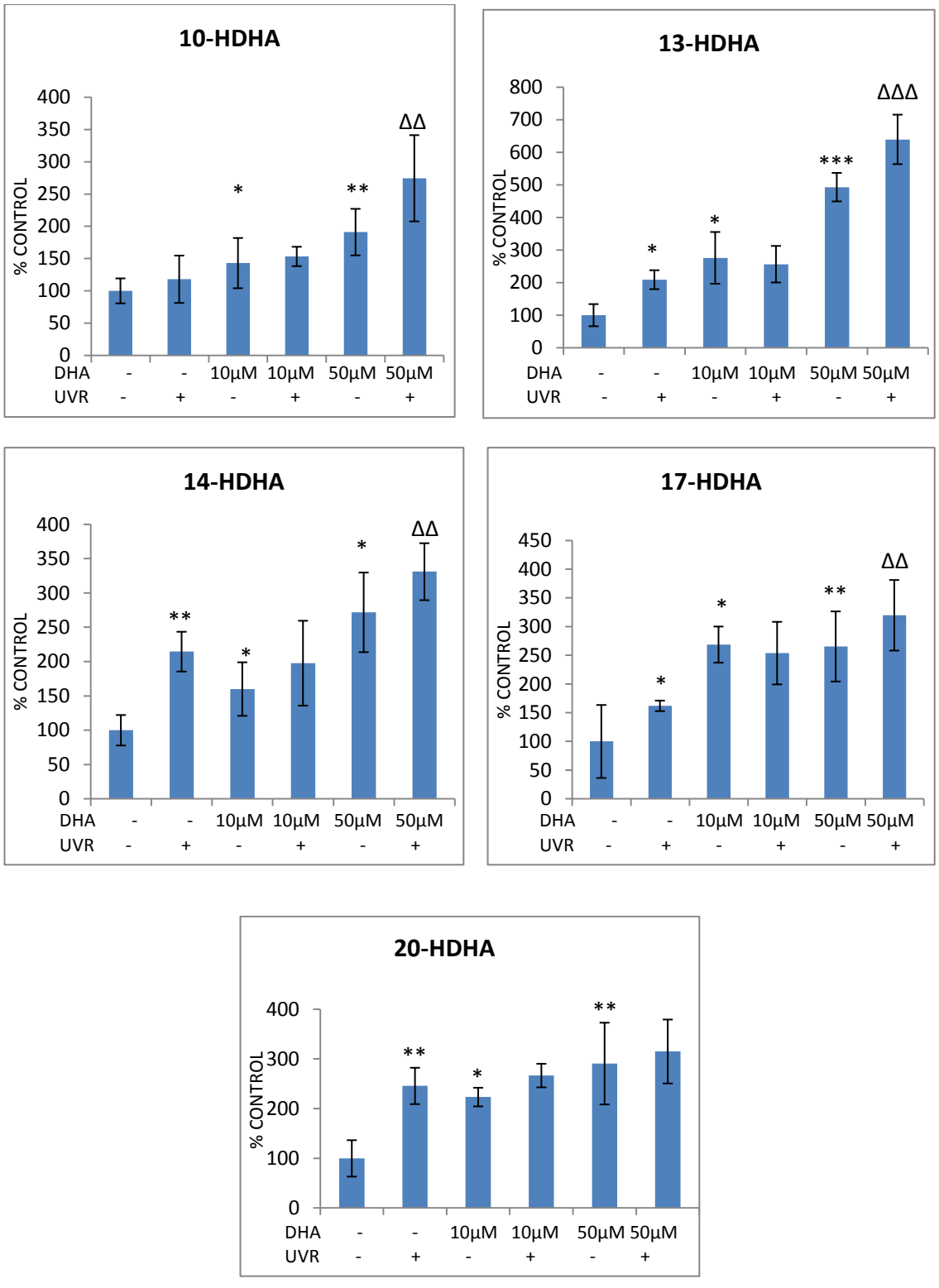


Figure 7.10. Effect of UVR and docosahexaenoic acid (DHA) treatment on hydroxy-docosahexaenoic acid (HDHA) mediators produced by HaCaT keratinocytes 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10μM and 50 μM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *p≤0.05, **p≤0.01, *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

7.3.2.5. The effect of UVR and fatty acid treatment on 14(15)-EET and 14, 15-DHET

As shown in Figure 7.12, the level of 14,15-EET and 14,15-DHET did not change when HaCaT cells were exposed to 15mJ/cm^2 UVR and compared to non-irradiated control (FA(-)/UVR(-)).

HaCaT cells treated with OA (10 and 50μM) showed no significant effect in 14(15)-EET and 14,15-DHET levels at baseline when compared to non-irradiated control (FA(-)/UVR(-)). Also OA had no effect on 14,(15)-EET and 14,15-DHET post UVR compared to irradiated control (FA (-)/UVR(+)).(Figures 7.11).

HaCaT treated with 10 and 50μM EPA did not have significantly different levels of 14(15)-EET and 14,15-DHET at baseline, compared to non-irradiated control (FA(-)/UVR(-)). Also no significant increase in 14(15)-EET and 14,15-DHET was found when HaCaT were treated to EPA and exposed to 15mJ/cm^2, compared to irradiated control (FA (-)/UVR(+)).(Figures 7.11).

Finally, HaCaT treated with 10 and 50μM DHA showed no effect on 14(15)-EET and 14,15-DHET at baseline compared to non-irradiated control (FA(-)/UVR(-)). Also, when exposed to 15mJ/cm^2 compared to irradiated control (FA (-)/UVR(+)).Result are shows in Figure 7.11.

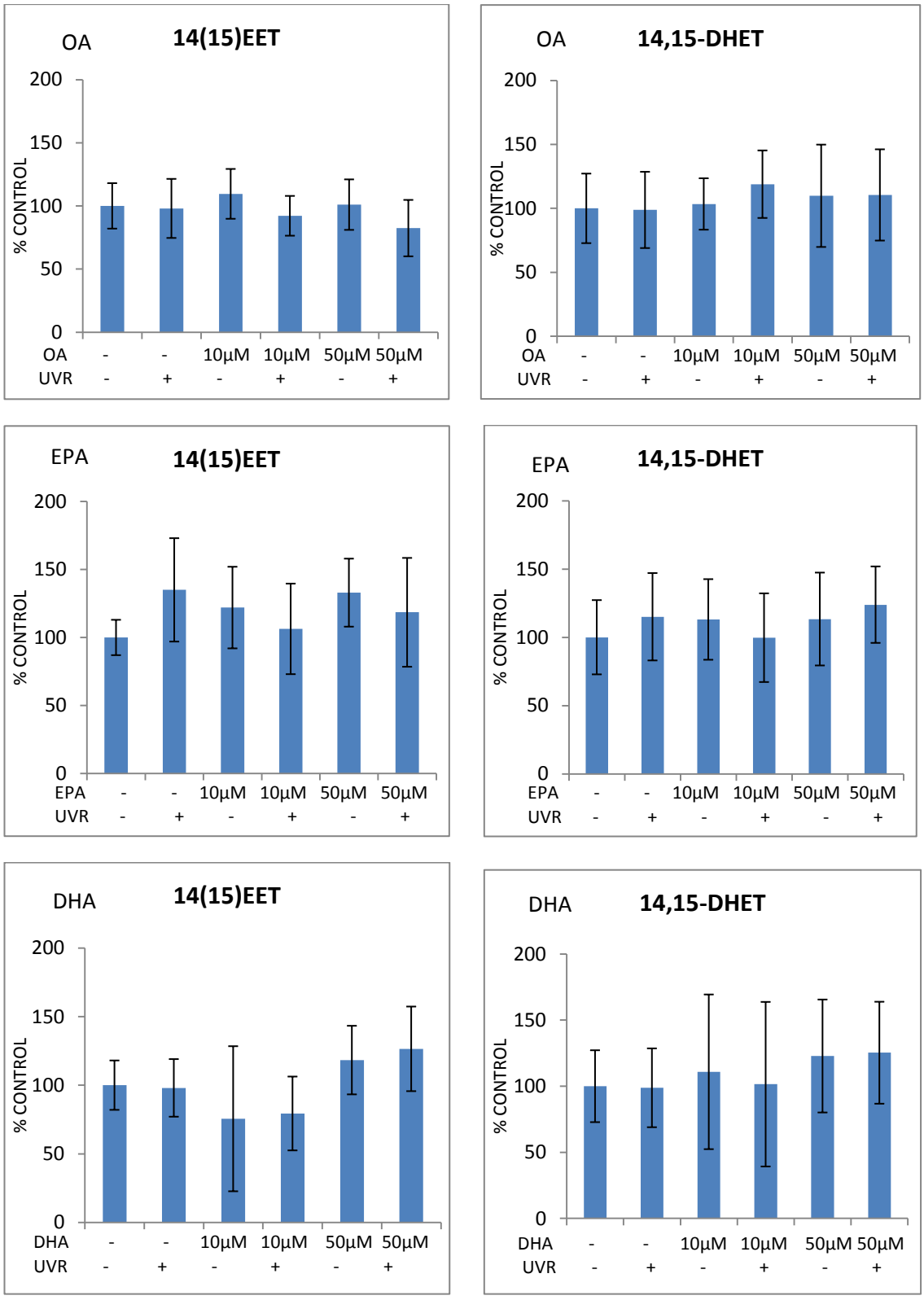


Figure 7.11. Effect of UVR and fatty acid treatment, oleic acid (OA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on 14(15)EET AND 14,15-DHET produced by HaCaT keratinocytes 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10μM and 50 μM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate).

7.3.2.6. The effect of UVR and fatty acid treatment on 5-oxo-ETE and 15-HETrE

As shown in Figure 7.12, the levels of 5-oxo-ETE were not statistically different when HaCaT cells were exposed to 15mJ/cm^2 UVR. While a significant increased of 15-HETrE ($p \leq 0.05$) was observed when compared to non-irradiated control (FA(-)/UVR(-)).

HaCaT cells treated with OA 10 and 50 μM did not show any significant change on 5-oxo-ETE levels at baseline or post UVR when compared to corresponding control groups. Also, OA had no significant effect on 15-HETrE (Figure 7.13).

HaCaT treated with 10 and 50μM EPA, showed some increase in 5-oxo-ETE at the baseline and post UVR, but this was not statistically significant. Also no significant changes in 15-HETrE levels at baseline. 50 μM EPA shown significant decreased in 15-HETrE ($p \leq 0.05$) post UVR compared to irradiated control (FA (-)/UVR(+)).Figure 7.13.

Finally HaCaT treated with 10 and 50μM DHA showed no significant difference on 5-oxo-ETE and 15-HETrE at baseline when compared to non-irradiated control (FA(-)/UVR(-)). While 50 μM DHA shown significant decreased in 15-HETrE ($p \leq 0.05$) post UVR compared to irradiated exposed to 15mJ/cm^2 compared to irradiated control (FA (-)/UVR(+)).Figure 7.13.

Summary of the effect of UVR and n-3 PUFA treatment on the hydroxy fatty acids mediators produced by HaCaT keratinocyte is shown in Table (7.2).

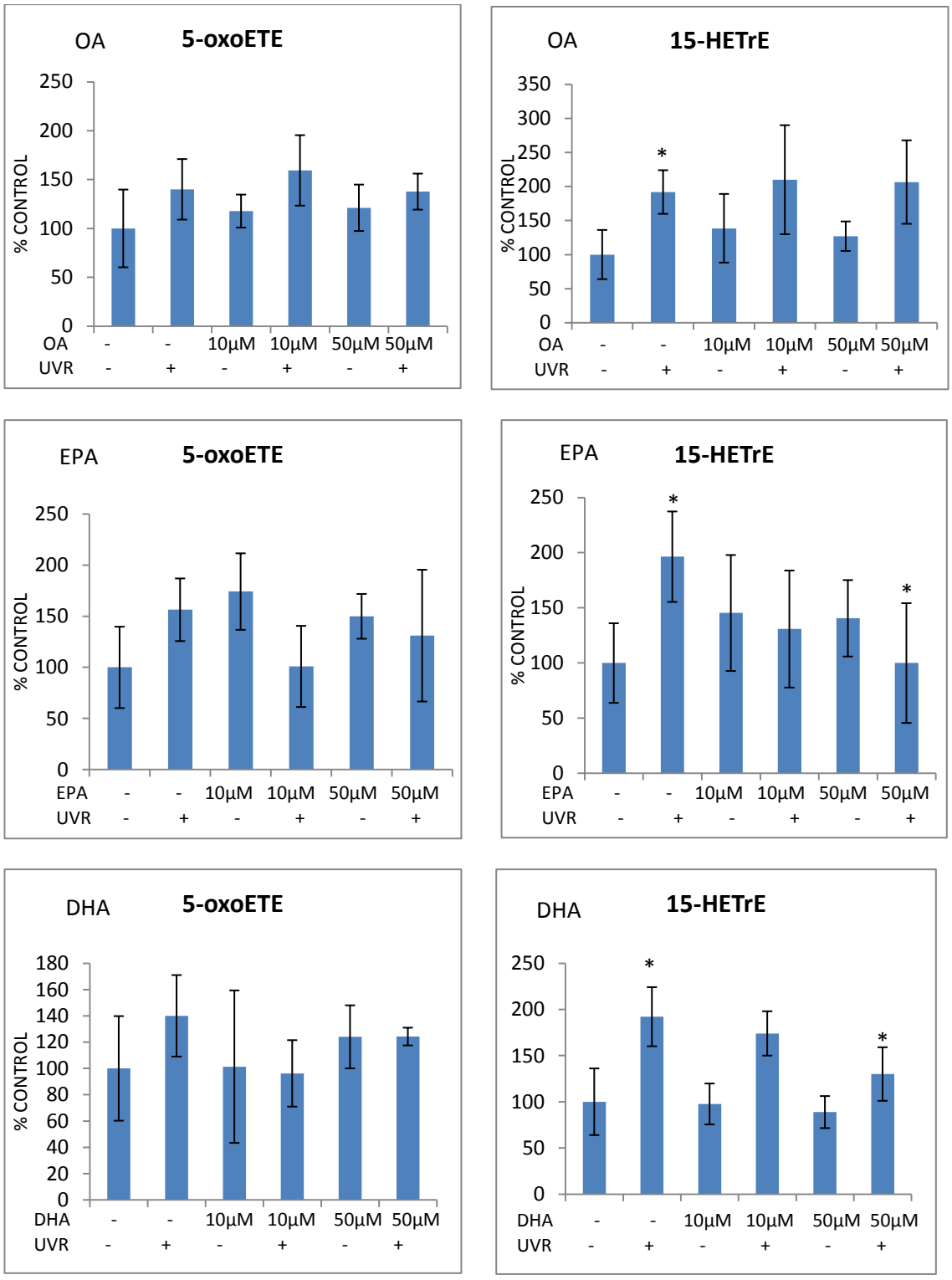


Figure 7.12. Effect of UVR and fatty acid treatment, oleic acid (OA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) treatment on 5-oxoETE and 15-HETrE produced by HaCaT keratinocytes 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate).

Table 7.2 Summary of the effect of UVR and n-3 PUFA treatment on the hydroxy fatty acids mediators produced by HaCaT keratinocyte

	UVR (15mJ/cm^2)	No UVR		UVR (15mJ/cm^2)	
		EPA	DHA	EPA	DHA
9- HODE	↑*	—	—	↓Δ	↓Δ
13-HODE	↑*	—	—	↓Δ	↓Δ
5- HEPE	↑*	↑*	↑*	↑Δ	—
12- HEPE	↑*	↑*	↑*	—	↑Δ
15- HEPE	↑*	—	—	↑Δ	—
18- HEPE	↑*	↑*	↑*	—	—
5- HETE	↑*	—	↓*	↓Δ	↓Δ
12- HETE	↑*	—	—	—	—
15- HETE	↑*	—	—	—	↓Δ
14- HDHA	↑*	—	↑*	—	↑Δ
17- HDHA	↑*	↑*	↑*	—	↑Δ

—= No significant effect

↓= Significant decrease

↑= Significant increase

*= compared to untreated cells (FA(-)/UVR (-))

Δ= compared to untreated irradiated cells (FA(-)/UVR (+))

7.3.3. The effect of UVR and fatty acid treatment on prostanoids produced by 46BR.1N fibroblasts

7.3.3.1. The effect of UVR and fatty acid treatment on HEPE

46BR.1N cells treated with 15mJ/cm^2 UVR showed significant increased in the level of 9-HODE ($p \leq 0.01$) and 13-HODE ($p \leq 0.05$) when compared to non-irradiated control (FA(-)/UVR(-)).Results are shown in Figure 7.13.

46.BR.1N cells treated with OA 10 and 50μM showed no significant changes in 9- and 13-HODE production at the baseline when compared to non-irradiated control (FA(-)/UVR(-)). Also, OA had no effect on 9-HODE and 13-HODE levels post UVR, compared to irradiated control (FA (-)/UVR(+)).(Figure 7.13).

46BR.1N treated with 10 and 50μM EPA showed no significant decrease on 9-HODE and 13-HODE levels at baseline compared to non-irradiated control (FA(-)/UVR(-)). Also, no significant decreased on 9-HODE or 13-HODE was found when 46.BR.1N were treated with 10 and 50μM EPA and exposed to 15mJ/cm^2 compared to irradiated control (FA (-)/UVR(+)).(Figures 7.13).

Finally, 46.BR.1N treated with 10 and 50μM DHA showed no significant decreased of 9-HODE. However, the level of 13-HODE was significant decreased ($P \leq 0.05$) when cells were treated with 50μM DHA at baseline compared, to non-irradiated control (FA(-)/UVR(-)). Also, 10 and 50μM DHA shown significant decrease of 9-HODE ($p \leq 0.05$, $p \leq 0.05$, respectively) and 13-HODE ($p \leq 0.05$, $p \leq 0.05$, respectively) levels post 15mJ/cm^2 compared to irradiated control (FA (-)/UVR(+)).Result shown in Figures 7.13.

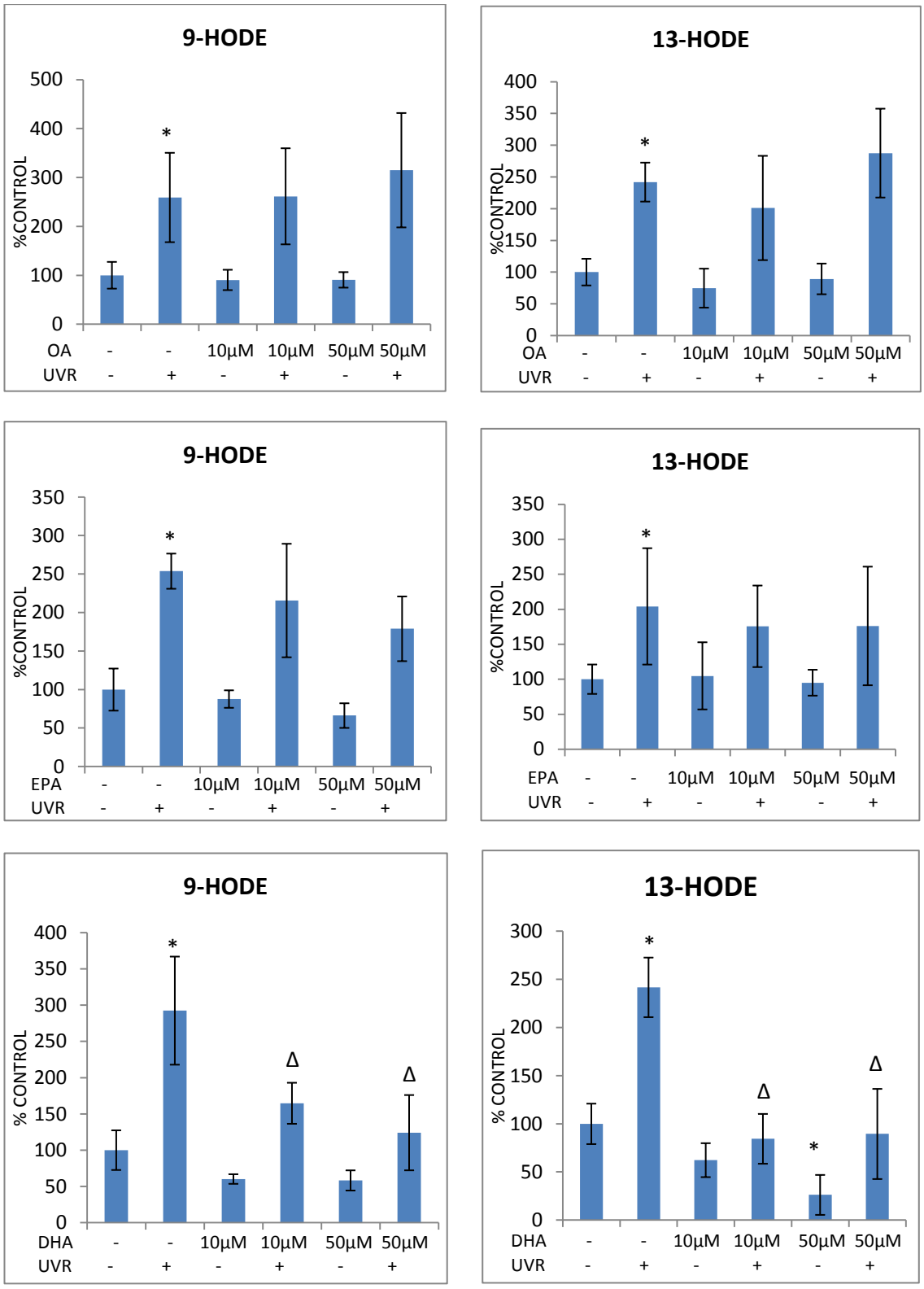


Figure 7.13. Effect of UVR and fatty acid treatment, oleic acid (OA), eicosapentaenoic acid(EPA) and docosahexaenoic acid (DHA) on HODE produced by 46BR.1N fibroblasts 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10μM and 50 μM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *Δp≤0.05, *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/ UVR(+)).

7.3.3.2. The effect of UVR and fatty acid treatment on HEPE

Exposure of the 46BR.1N cells to 15mJ/cm^2 UVR showed significant increase in the level of 5-,8-,9-,11-,12,15 and 18-HEPE (p≤0.05, 0.05, 0.001, 0.01, 0.01, 0.05 and 0.01; respectively) when compared to non-irradiated control (FA(-)/UVR(-)).

As shown in Figure 7.14 there was no effect of OA (10 and 50 μM) on the level of HEPE mediators at the baseline or post UVR when compared to corresponding control

46BR.1N treated with 10 and 50μM EPA showed significant increase of 5-,8-, 9-,11-,12-,15- and 18-HEPE ((p≤0.05,0.05), (p≤0.01, 0.01), (p≤0.01, 0.01), (p≤01,0.01), (p≤0.05, 0.001), (p≤0.05, 0.001) and (p≤0.01, 0.001);respectively). Also significant increase of 5-, 8-, 9-,11, 12-, 15- and 18-HEPA (p≤0.05,0.01, p≤0.05,0.001, p≤0.01, 0.001, p≤0.01,0.01, p≤0.01, 0.001, p≤0.01, 0.01, and p≤0.05,0.001; respectively) was observed when 46BR.1N cells were treated with 10 and 50 μM of EPA and exposed to 15 mJ/cm^2 UVR compared to corresponding control.(Figure 7.14).

46BR.1N treated with 10 and 50μM DHA showed significant increase of 5-,8-,11-,12- and 18-HEPE (p≤0.05,0.01, p≤0.01,0.01 p≤0.01, 0.01, p≤0.05,0.01 and p≤0.05,0.01;respectively). While, 9- and 15-HEPE (p≤0.01 and p≤0.01; respectively) was significant increase post 50μM DHA. Also The level of 8-,12- and 18-HEBE (p≤0.01,0.01, p≤0.01,0.01 and p≤0.05,0.01; respectively) was significant increase when cells treated with 10 and 50μM post 15mJ/cm^2. 5- and 15-HEBE (p≤0.01) was significant increase post 50 μM compared to non-irradiated control (FA(-)/UVR(-)) (Figure 7.22).

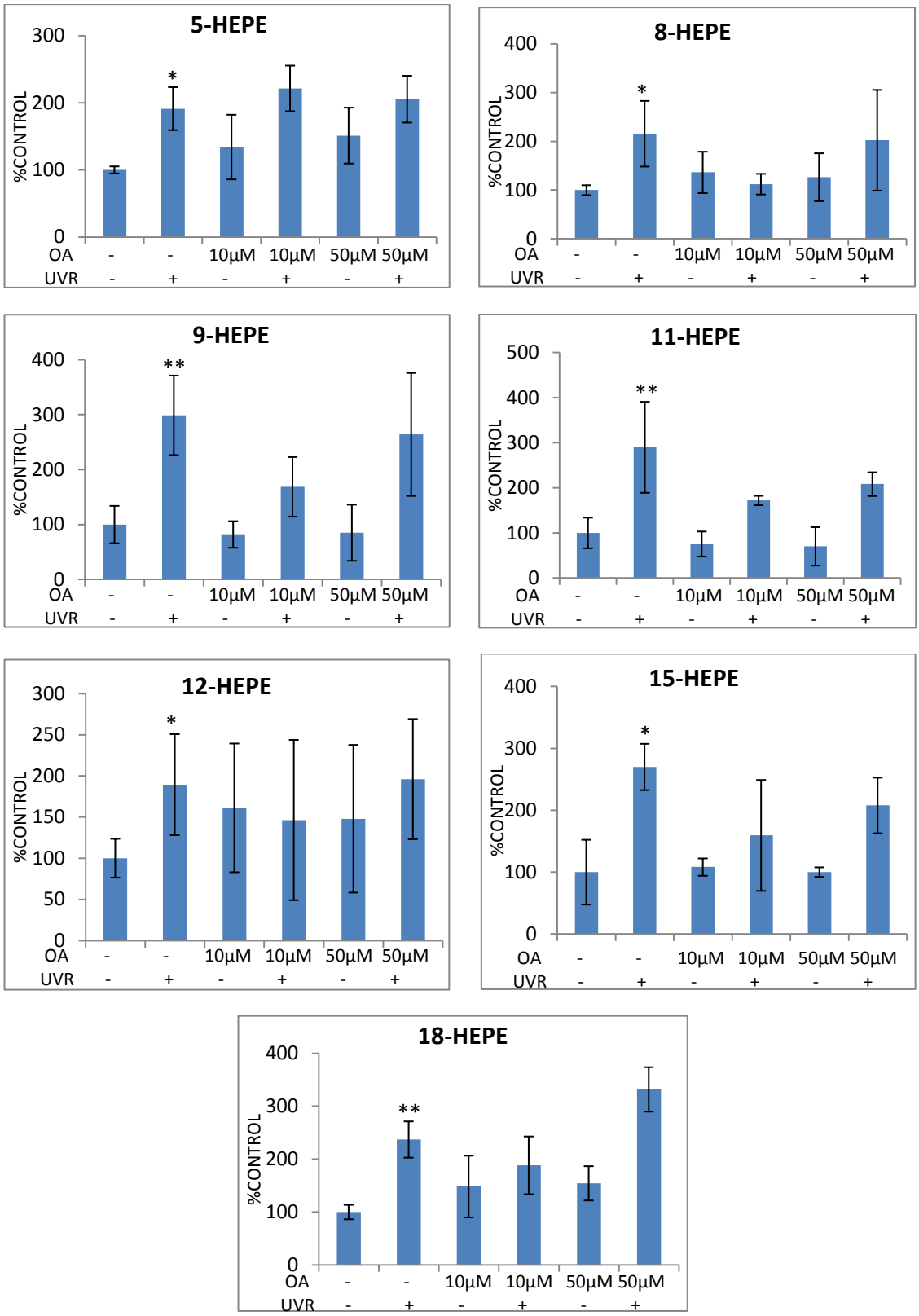


Figure 7.14. Effect of UVR and oleic acid (OA) treatment on hydroxy-eicosapentaenoic acid (HEPE) produced by 46BR.1N fibroblasts 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10μM and 50 μM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *p≤0.05, **p≤0.01, *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

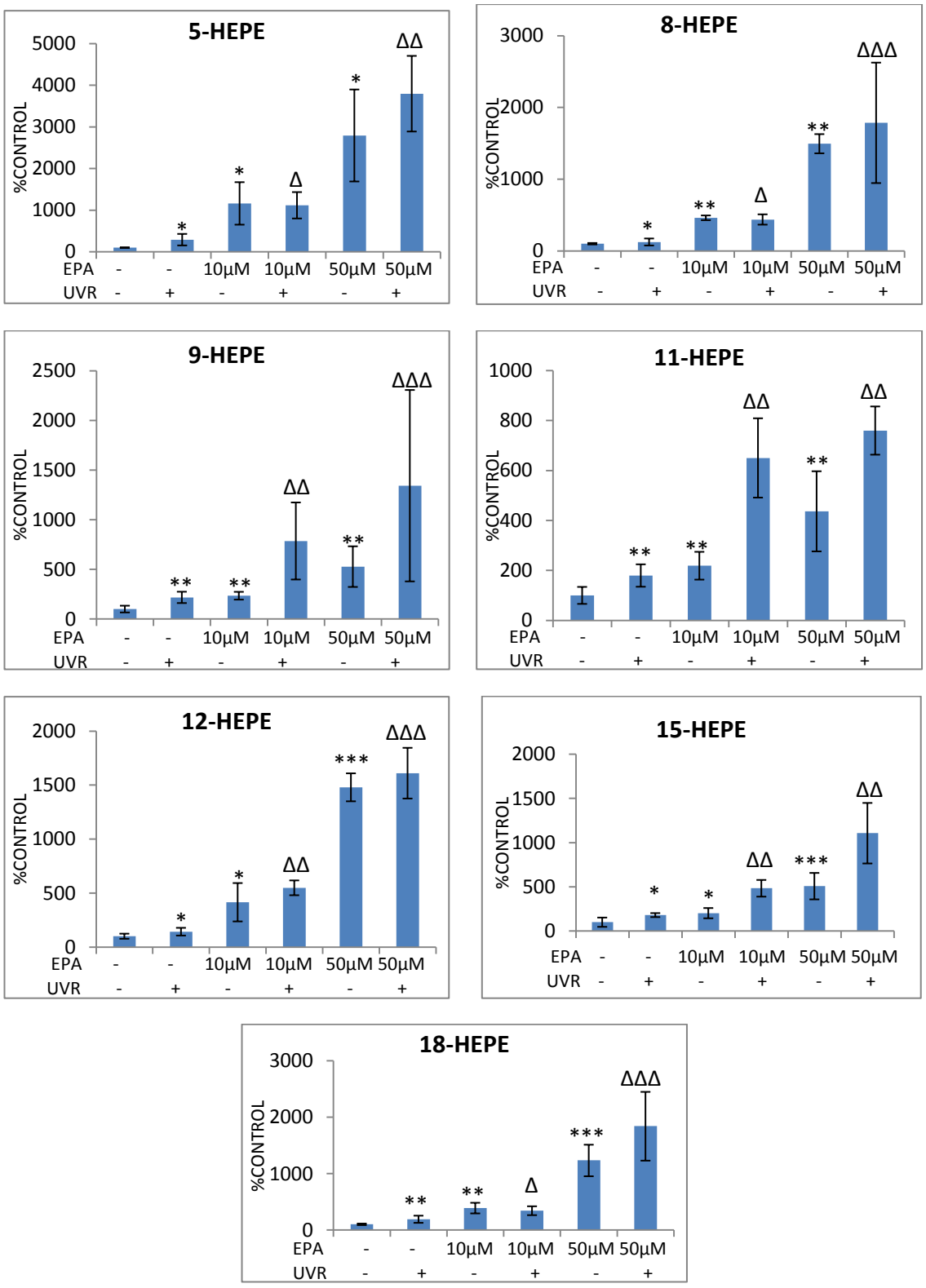


Figure 7.15. Effect of UVR and eicosapentaenoic acid (EPA) treatment on hydroxy-eicosapentaenoic acid (HEPE) mediators produced by 46BR.1N fibroblasts 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10μM and 50 μM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *Δp≤0.05, **ΔΔp≤0.01, ***ΔΔΔp≤0.001 *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

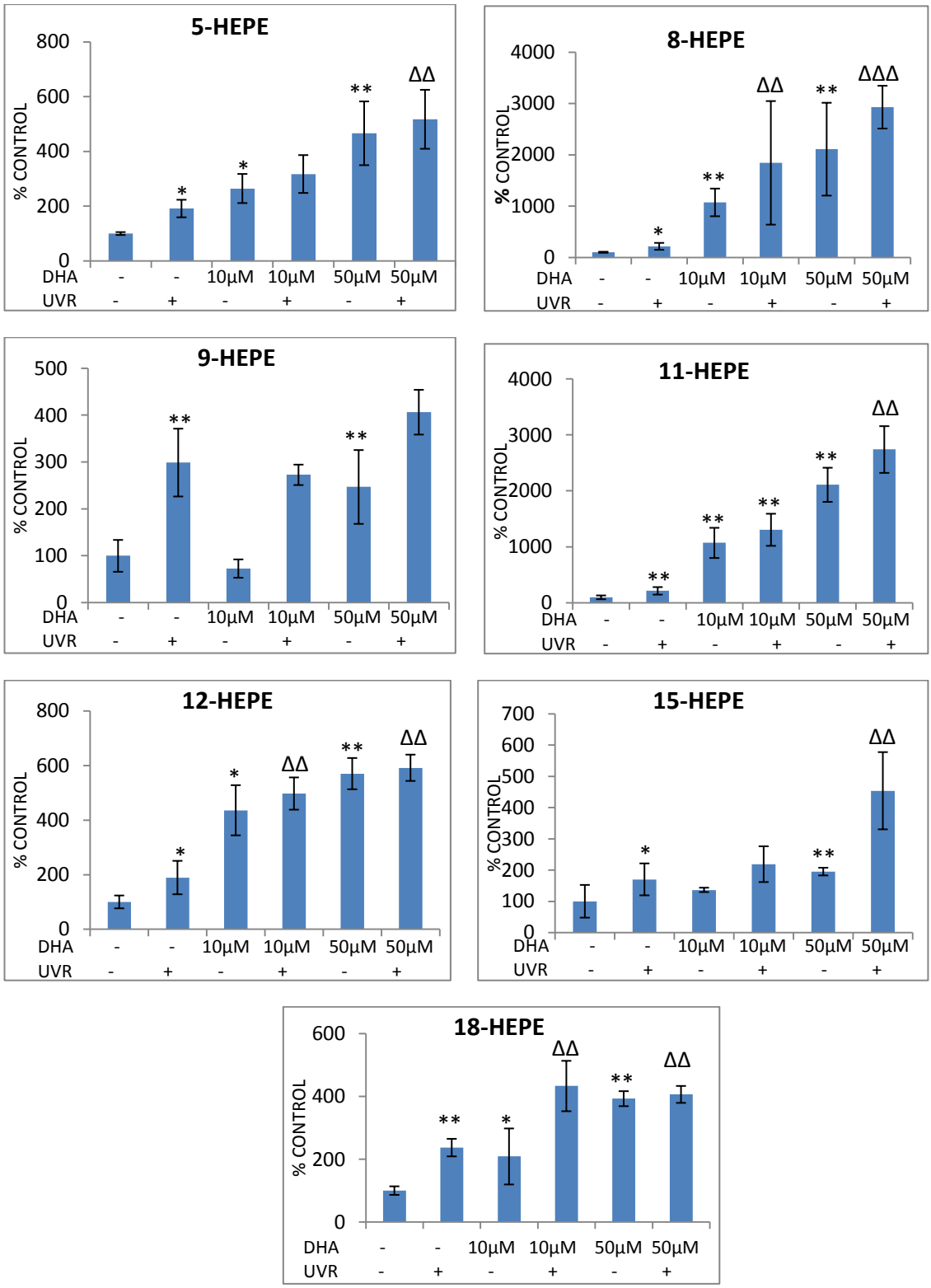


Figure 7.16. Effect of UVR and docosahexaenoic acid (DHA) treatment on hydroxy-eicosapentaenoic acid (HEPE) mediators produced by 46BR.1N fibroblasts 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10μM and 50 μM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *Δp≤0.05, **ΔΔp≤0.01, ***ΔΔΔp≤0.001 *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

7.3.3.3. The effect of UVR and fatty acid treatment on HETE

Six HETE mediators were detected in 46BR.1N cells. Exposure of the cells to 15mJ/cm^2 UVR resulted in a significant increase in 5-, 8-, 9-, 11-, 12- and 15-HETE ($p \leq 0.01$, 0.05, 0.01, 0.01, 0.05 and 0.05; respectively) when compared to non-irradiated control (FA(-)/UVR(-)).

Figure 7.17 showed the effect of OA (10 and 50 μM) on the levels of HETE mediators at baseline, and following exposure to UVR. No statistically significant changes were observed when the data was compared to irradiated control (FA (-)/UVR(+)).

No significant decrease in 46BR.1N cell HETE mediators was observed when the cells were treated with 10 and 50 μM of EPA compared to non-irradiated control (FA(-)/UVR(-)). Moreover, a significant decrease in 12-HETE ($p \leq 0.05$) and 15-HETE, ($p \leq 0.05$) was observed when 46BR.1N cells were treated with 50 μM of EPA and exposed to 15 mJ/cm^2 UVR. Compared to irradiated control (FA (-)/UVR(+)).(Figure 7.18)

Finally 46BR.1N treated with 10 and 50μM DHA showed no significant decreased in HETE mediators at baseline when compared to non-irradiated control (FA(-)/UVR(-)). Moreover, significant decrease in 9-, 11-, 12- and 15-HETE ($p \leq 0.01$, 0.01, $p \leq 0.01$, 0.01, $p \leq 0.05$, 0.01 and $p \leq 0.01$, 0.01; respectively) was observed when 46BR.1N cells were treated with 10 and 50 μM of DHA, exposed to 15 mJ/cm^2 UVR and compared to irradiated control (FA (-)/UVR(+)).

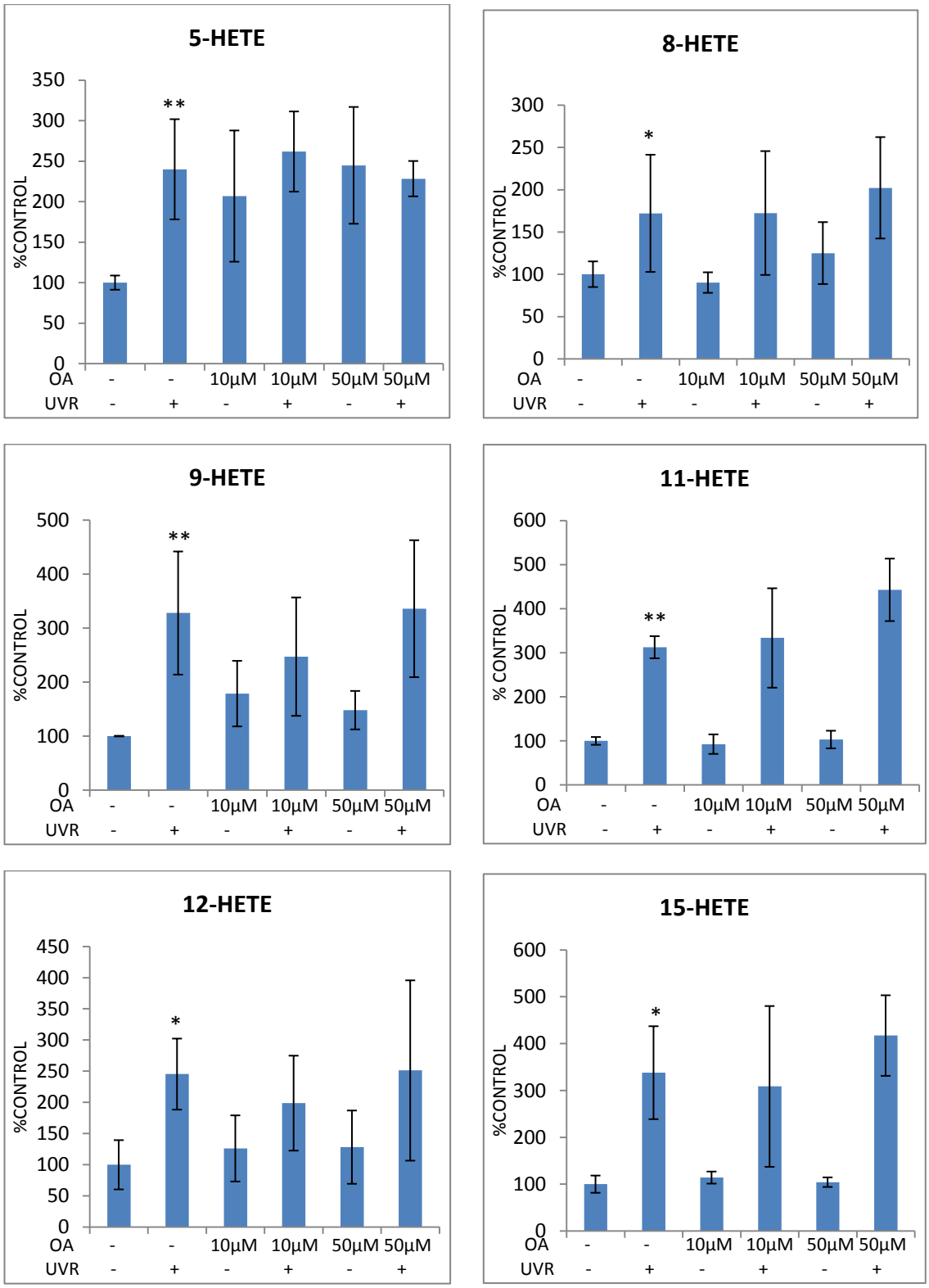


Figure 7.17. Effect of UVR and oleic acid (OA) treatment on hydroxy-eicosatetraenoic acid (HETE) mediators produced by 46BR.1N fibroblasts 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10μM and 50 μM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *p≤0.05, **p≤0.01, *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

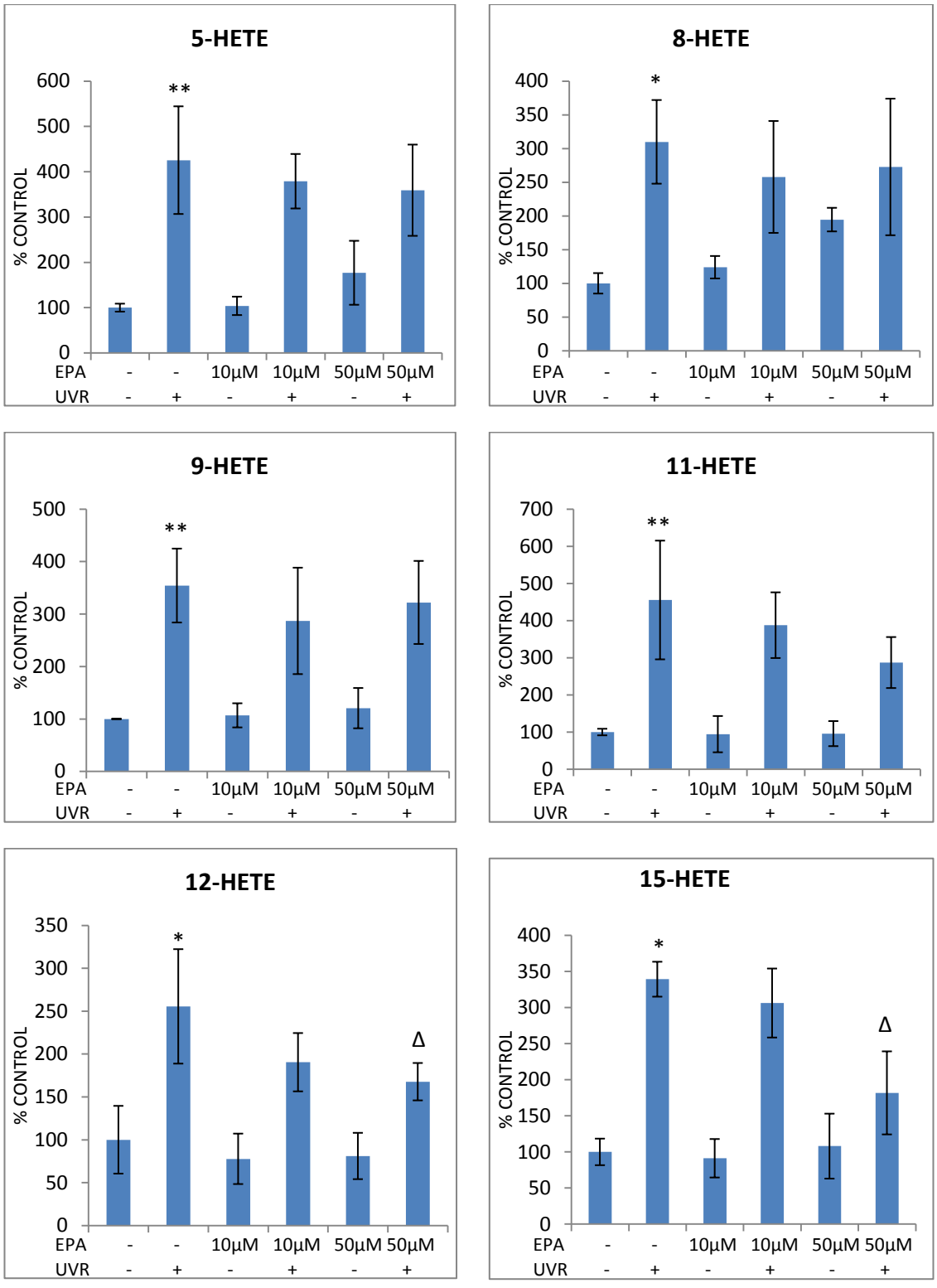


Figure 7.18. Effect of UVR and eicosapentaenoic acid (EPA) treatment on hydroxy-eicosatetraenoic acid (HETE) mediators produced by 46BR.1N fibroblasts 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *Δp≤0.05, **p≤0.01, *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

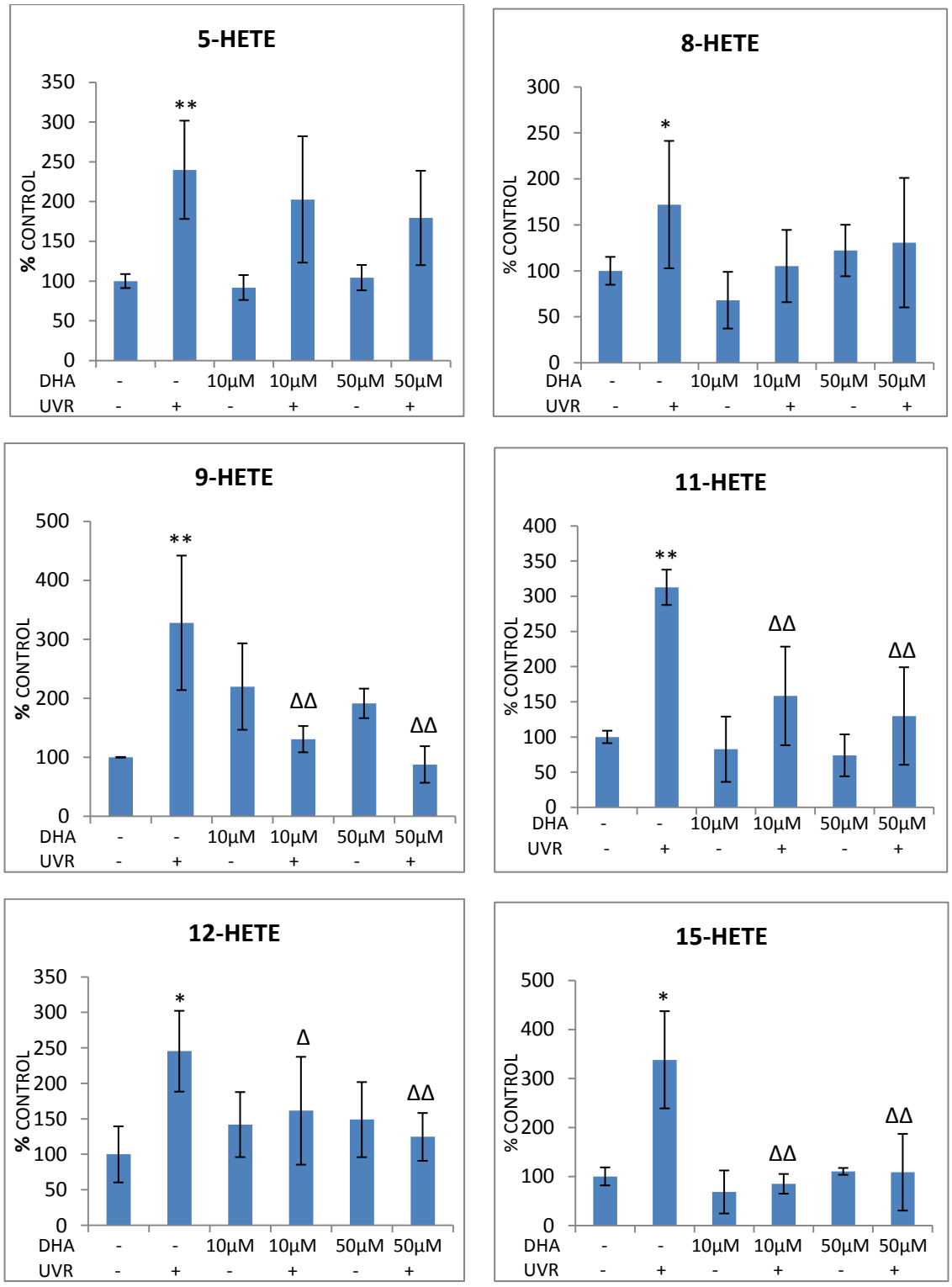


Figure 7.19. Effect of UVR and docosahexaenoic acid (DHA) treatment on hydroxy-eicosatetraenoic acid (HETE) mediators produced by 46BR.1N fibroblasts 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10μM and 50 μM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *Δp≤0.05, **ΔΔp≤0.01, *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

7.3.3.4. The effect of UVR and fatty acid treatment on HDHA

Five HDHA mediators were detected in 46BR.1N cells. Following exposure to 15mJ/cm^2 UVR, significant increase in the level of 10-, 13-,14-,17- and 20-HDHA ($p \leq 0.05$, $p \leq 0.05$, $p \leq 0.05$, $p \leq 0.05$ and $p \leq 0.01$; respectively) were detected when compared to non-irradiated control (FA(-)/UVR(-)).

Figure 7.20 shows the effect of OA (10 and 50 µM) on the level of HDHA mediators at baseline, and there was no significant difference when compared to non-irradiated control (FA(-)/UVR(-)). Also no significant difference on HDHA mediators was found when 46BR.1N cells that were treated with OA (10 and 50 µM) were exposed to 15mJ/cm^2 compared to irradiated control (FA (-)/UVR(+)).

The results shown in Figure 7.21 present the effect of EPA treatment (10 and 50 µM) on the level of HDHA mediators on 46BR.1N cells. Although there was increase in the level of these mediators after the treatment, this was not statistically significant when compared to non-irradiated control (FA(-)/UVR(-)). Moreover, an increase these mediators was observed when 46BR.1N cells were treated with 10 and 50 µM of EPA and exposed to 15 mJ/cm^2 UVR but this was not statistically significant compared to irradiated control (FA (-)/UVR(+)).

The level of 10-, 13-,14-,17- and 20-HDHA were significant increased ($P \leq 0.01$, 0.001, $P \leq 0.05$, 0.001, $P \leq 0.05$, 0.01, $P \leq 0.05$, 0.001 and $P \leq 0.01$, 0.001; respectively) when 46BR.1N cells were treated with 10 and 50 µM of DHA compared to non-irradiated control (FA(-)/UVR(-)). Also, the level of 10-,14-,17- and 20-HDHA ($P \leq 0.01$, 0.001, $P \leq 0.01$, 0.01, $P \leq 0.05$, 0.01 and $P \leq 0.01$, 0.01; respectively) was significant increase post 15mJ/cm^2 compared to non-irradiated control (FA(-)/UVR(-)) (Figure 7.22).

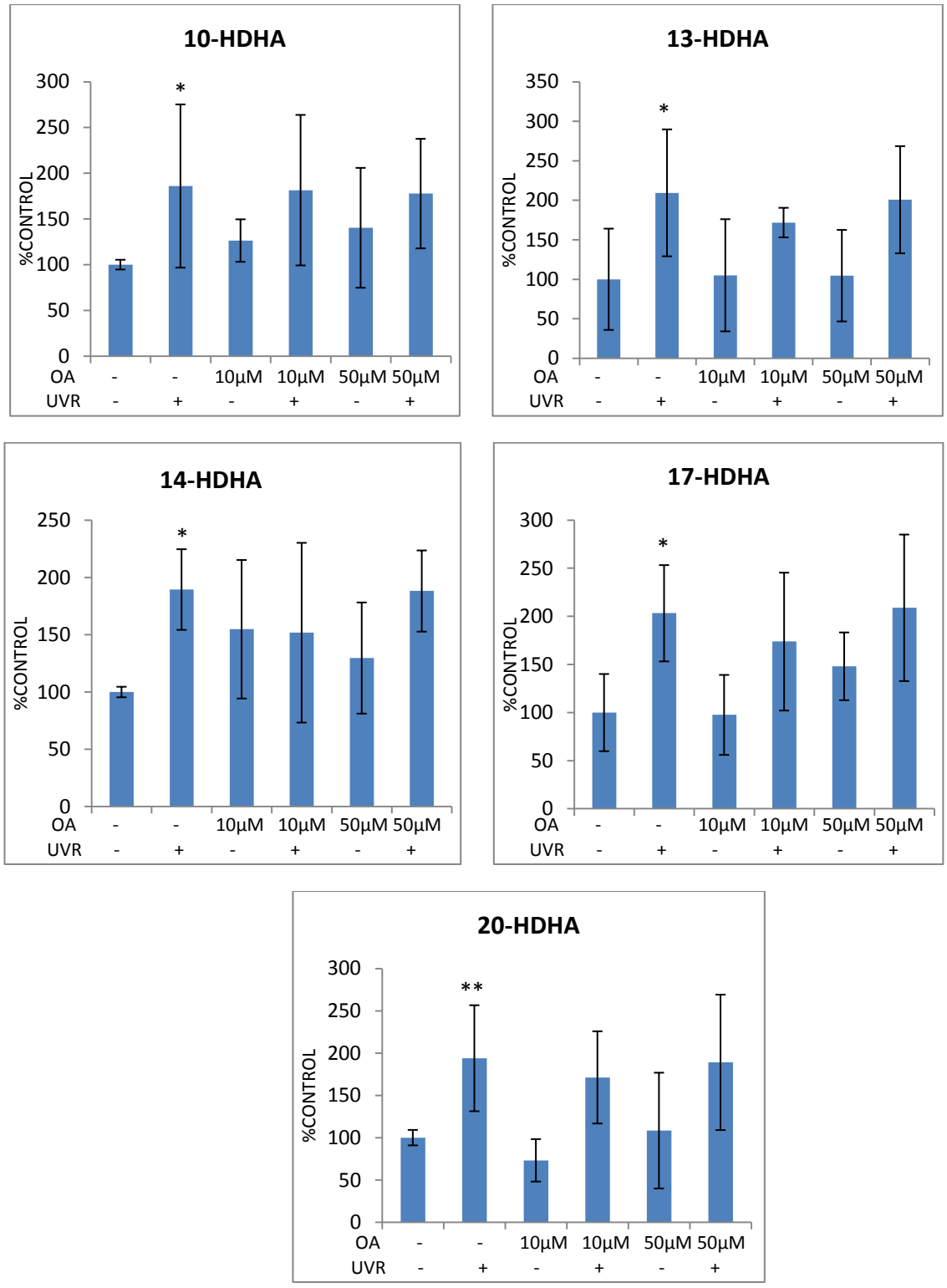


Figure 7.20. Effect of UVR and oleic acid (OA) treatment on hydroxy-docosahexaenoic acid (HDHA) mediators produced by 46BR.1N fibroblasts 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10μM and 50 μM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *p≤0.05, **p≤0.01, *=compared to non-irradiated control (FA(-)/UVR(-)).

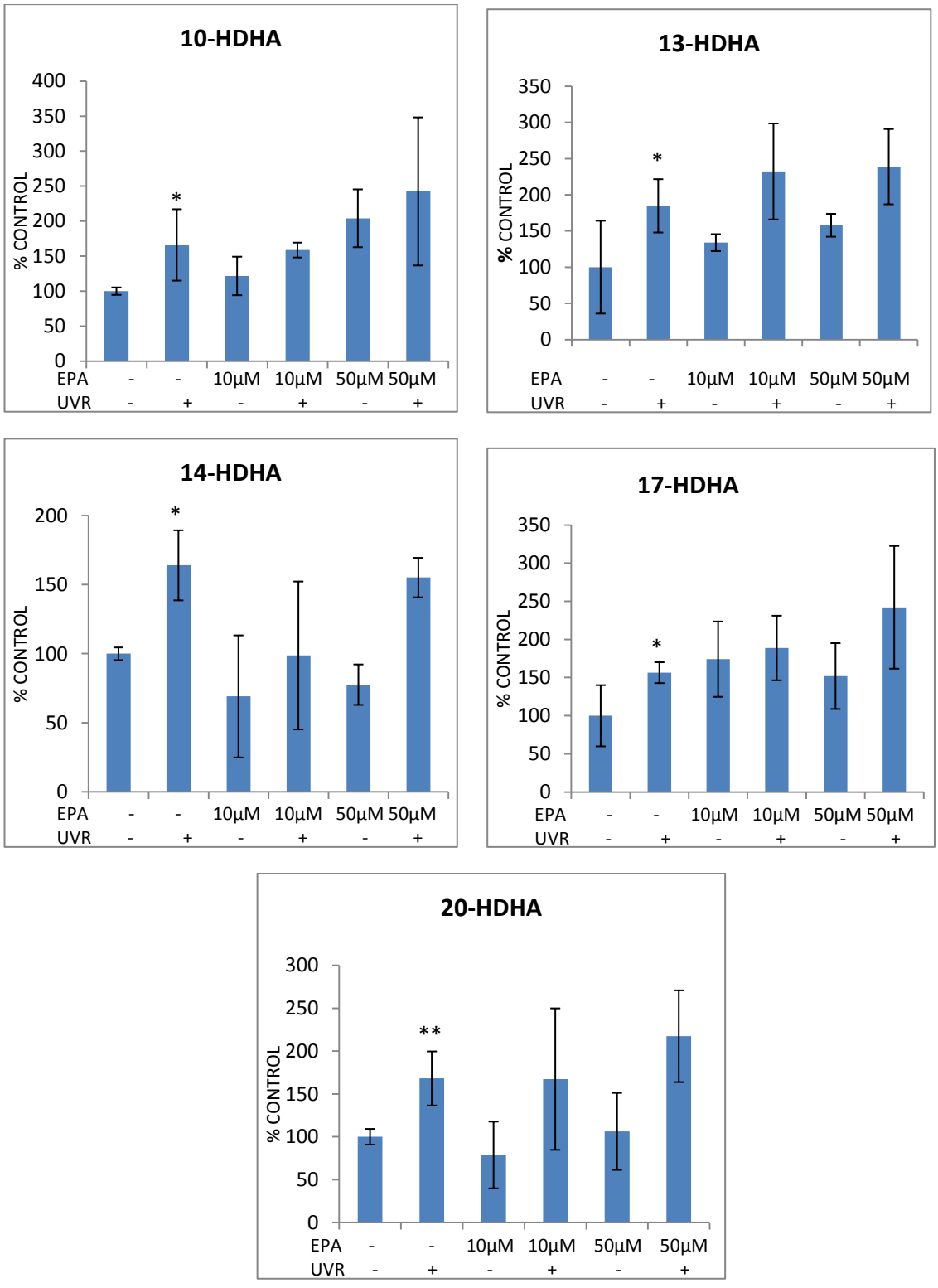


Figure 7.21. Effect of UVR and eicosapentaenoic acid (EPA) treatment on hydroxy-docosahexaenoic acid (HDHA) mediators produced by 46BR.1N fibroblasts 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10µM and 50 µM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *p≤0.05, **p≤0.01, *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

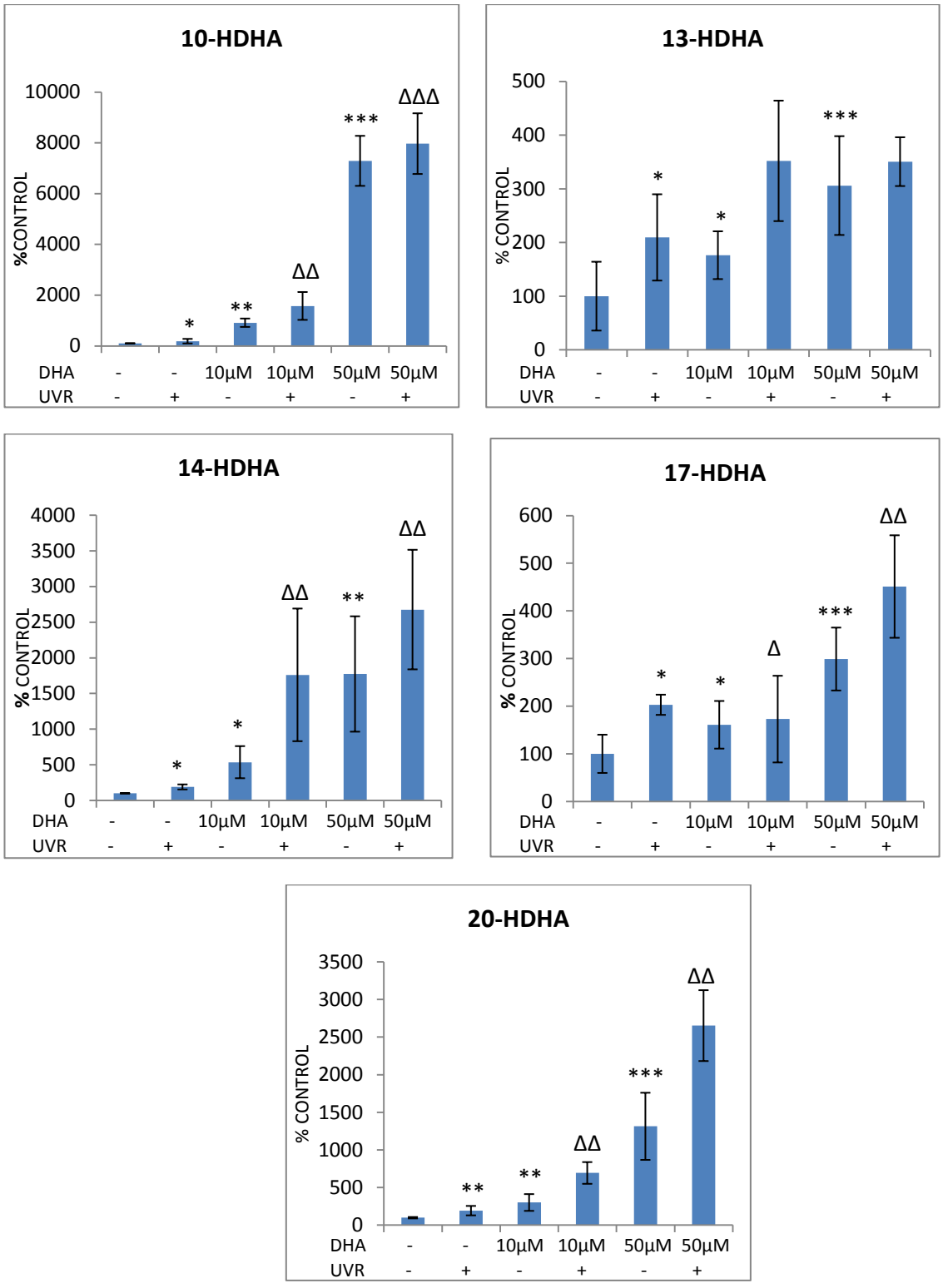


Figure 7.22. Effect of UVR and docosahexaenoic acid (DHA) treatment on hydroxy-docosahexaenoic acid (HDHA) mediators produced by 46BR.1N fibroblasts 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10μM and 50 μM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *Δp≤0.05, **ΔΔp≤0.01, ***ΔΔΔp≤0.001 *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

7.3.3.5. The effect of UVR and fatty acid treatment on 14(15)-EET and 14, 15-DHET

As shown in Figure 7.23, the level of 14(15)-EET ($p \leq 0.05$) and 14,15-DHET ($p \leq 0.05$) were significantly increased when HaCaT cells were exposed to 15mJ/cm^2 UVR and compared to non-irradiated control (FA(-)/UVR(-)).

46BR.1N cells treated with OA (10 and 50μM) showed no significant change in 14(15)-EET and 14,15-DHET levels at baseline when compared to non-irradiated control (FA(-)/UVR(-)). Also OA had no effect on 14,(15)-EET and 14,15-DHET post UVR compared to irradiated control (FA (-)/UVR(+)).(Figures 7.23).

46BR.1N treated with 10 and 50μM EPA did not have significantly different levels of 14(15)-EET and 14,15-DHET at baseline, compared to non-irradiated control (FA(-)/UVR(-)). Also no significant increase in 14(15)-EET and 14,15-DHET was found when 46BR.1N were treated to EPA and exposed to 15mJ/cm^2, compared to irradiated control (FA (-)/UVR(+)).(Figure 7.23).

Finally, 46BR.1N treated with 10 and 50μM DHA showed no change in 14(15)-EET and 14,15-DHET at baseline compared to non-irradiated control (FA(-)/UVR(-)). Also, when exposed to 15mJ/cm^2 compared to irradiated control (FA (-)/UVR(+)). Results are shown in Figure 7.23.

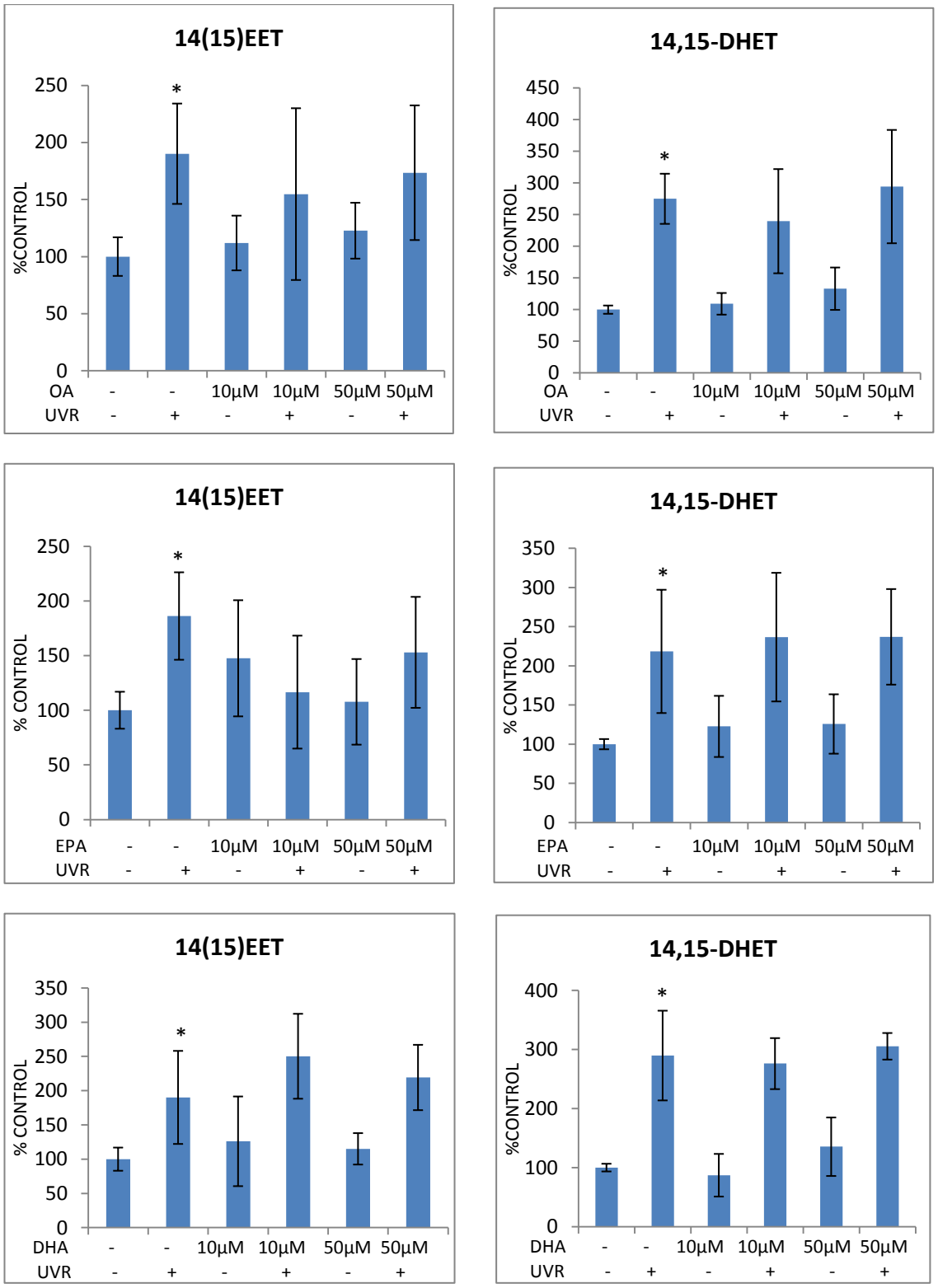


Figure 7.23. Effect of UVR and fatty acid treatment, oleic acid (OA), eicosapentaenoic acid(EPA) and docosahexaenoic acid (DHA) on 14(15)EET and 14,15-DHET produced by 46BR.1N fibroblasts 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10μM and 50 μM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *p≤0.001 *=compared to non-irradiated control (FA(-)/UVR(-)).

7.3.3.6. The effect of UVR and fatty acid treatment on 5-OXO-ETE and 15-HETrE

As shown in Figure 7.12, the levels of 5-oxo-ETE ($p=0.013$) and 15-HETrE ($p=0.03$) were statistically increased when 46BR.1N cells were exposed to 15mJ/cm^2 UVR and compared to non-irradiated control (FA(-)/UVR(-)).

46BR.1N cells treated with OA 10 and 50μM did not shown any significant increase on 5-oxo-ETE levels at baseline or post UVR when compared to corresponding control groups. Also OA had no significant effect on 15-HETrE (Figures 7.24).

46BR.1N treated with 10 and 50μM EPA, showed some increase in 5-oxo-ETE at the baseline and post UVR, but this was not statistically significant. Also there were no significant changes in 15-HETrE levels at baseline. 50μM EPA showed significant decreased in 15-HETrE ($p\leq0.05$) post UVR compared to irradiated control (FA (-)/UVR(+)).Figures 7.24.

Finally 46BR.1N treated with 10 and 50μM DHA showed no significant difference on 5-oxo-ETE and 15-HETrE at baseline when compared to non-irradiated control (FA(-)/UVR(-)). A significant decrease in 15-HETrE ($p\leq0.05$) was observed when cells treated with 50μM DHA, were exposed to 15 mJ/cm^2 and compared to irradiated control (FA (-)/UVR(+)). Figure 7.24.

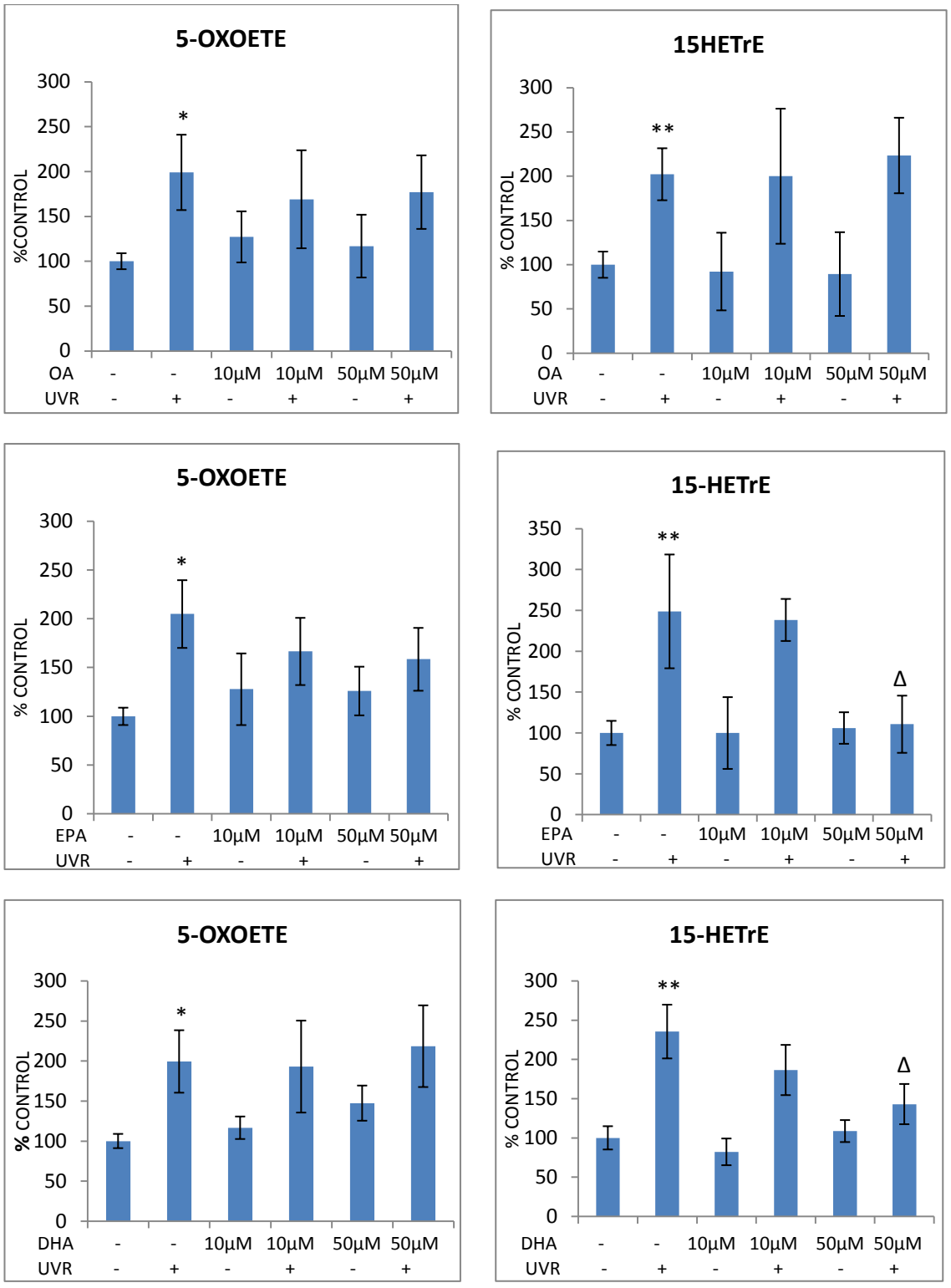


Figure 7.24. Effect of UVR and fatty acid treatment, oleic acid (OA), eicosapentaenoic acid(EPA) and docosahexaenoic acid (DHA) on 5oxoETE and 15-HETrE produced by 46BR.1N fibroblasts 24h post UVR (15 mJ/cm^2). Cells were treated with two concentrations of each fatty acid (10μM and 50 μM) for 72h. Results are shown as mean ± SD for 3 independent experiments (each one performed in duplicate). *Δp≤0.05 *=compared to non-irradiated control (FA(-)/UVR(-)). Δ=compared to irradiated control (FA (-)/UVR(+)).

Table 7.3. Summary of the effect of UVR and n-3 PUFA treatment on the hydroxy fatty acids mediators produced by 46BR.1N fibroblasts.

	UVR (15mJ/cm^2)	No UVR		UVR (15mJ/cm^2)	
		EPA	DHA	EPA	DHA
9- HODE	↑*	—	—	—	↓Δ
13-HODE	↑*	—	↓*	—	↓Δ
5- HEPE	↑*	↑*	↑*	↑Δ	↑Δ
12- HEPE	↑*	↑*	↑*	↑Δ	↑Δ
15- HEPE	↑*	↑*	↑*	↑Δ	↑Δ
18- HEPE	↑*	↑*	↑*	↑Δ	↑Δ
5- HETE	↑*	—	—	—	—
12- HETE	↑*	—	—	↓Δ	↓Δ
15- HETE	↑*	—	—	↓Δ	↓Δ
14- HDHA	↑*	—	↑*	—	↑Δ
17- HDHA	↑*	—	↑*	—	↑Δ

—= No significant effect

↓= Significant decrease

↑= Significant increase

*= compared to untreated cells (FA(-)/UVR (-))

Δ= compared to untreated irradiated cells (FA(-)/UVR (+))

7.4. Discussion

Data in this study have shown that a range of hydroxy fatty acids were produced from different fatty acid precursors, including LA, AA, EPA, DHA and DGLA in HaCaT and 46BR.1N. At baseline and in both cell lines, 13-HODE was the main hydroxy fatty acid generated from LA, 15-HEPE was the main HEPE generated form EPA, 12-HETE was the highest AA metabolite, and 17-HDHA was the main hydroxy fatty acid generated from DHA. Other hydroxy fatty acids were detected including 5-oxoETE, a product of dehydrogenation of 5-HETE and 15-HETrE from DGLA. In addition, the epoxide 14(15) EET and its metabolite 14,15-DHET were detected in both HaCaT and 46BR.1N cells. Overall, 13-HODE (15.7%, 28.3%) was found to be the most abundant hydroxy fatty acid in HaCaT and 46BR.1N cells followed by 12-HETE (12%, 11%) and 15-HEPE (9.3%, 8%). In agreement with this study 13-HODE was reported to be the main hydroxy fatty acid in epidermal guinea pigs (Miller et al., 1990). Many studies have reported that 12- HETE is the main pro-inflammatory mediator produced from AA in the skin (Rhodes et al., 2009). Moreover, 12-HETE has been found to be the main hydroxy fatty acid in human epidermal cells (Henneickevonzepelin et al., 1991). A study in human skin fibroblasts reported that only 4.5% of the lipid extract was monohydroxy fatty acids and this fraction contained 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) 33%, 15-HETE 31% and 11-HETE 22%. No other monohydroxy fatty acids were detected in that study, but this was an old paper whose method may not have been very sensitive (Mayer et al., 1984). The paper concluded that 15-HETE is the main LOX product in human skin fibroblasts, where 12-HETE is the main LOX product found in this study. The high levels of 13-HODE and 12-HETE

may reflect their precursors LA and AA, respectively, which are the most abundant PUFAs in the skin (Tables 3.2 and 5.1).

The epoxy fatty acid 14,(15)-EET was detected in HaCaT and 46BR.1N cells at low levels (2% and 0.6% respectively). The dihydroxy fatty acid 14, 15-DHET was also detected in HaCaT and 46BR.1N cells at 2% and 0.6% respectively. It is known that EETs are hydrated by the cytosolic epoxide hydrolase to give the inactive DHET (Zeldin et al., 1995). A study on human skin fibroblasts reported another metabolic pathway for 11,12- and 14,15-EET: EETs are converted to the chain-shortened epoxy-fatty acids of DHET by β-oxidation. They are produced when the fibroblasts are exposed to a relatively high concentration of EET. This suggests that partial β-oxidation, rather than hydration by epoxide hydrolase, is the main pathway for EET metabolism in human fibroblasts (Fang et al., 2000).

The present study has shown that exposing HaCaT and 46BR.1N cells to 15mJ/cm^2 UVR produced significant increases in most hydroxy fatty acids at 24h post UVR. Rhodes et al (2009) reported that 8, 11, 12 and 15-HETE were significantly increased post UVR in the skin of 32 healthy human volunteers (Rhodes et al., 2009). 12-HETE has also been found to be increased after UV exposure in a study of skin blister fluid in six subjects (Black et al., 1985) and in dermal microdialysate in three subjects (Grundmann et al., 2004). 12-LOX is the key enzyme producing 12-HETE. Exposure of HaCaT cells to UVB and UVA showed a dose-dependent decrease in the level of 12-LOX expression in HaCaT keratinocytes (Yoo et al., 2008).

The results of this study show that treatment of HaCaT and 46BR.1N with EPA led to an increase of most HEPE mediators and some HDHA mediators, and decreased HODE and HETE production, especially post UVR. EPA is the precursor of HEPE, and the increase in these mediators after EPA treatment reflects the increase in EPA content in HaCaT and 46BR.1N. Meanwhile, increased HDHA mediators after EPA treatment may reflect the conversion of EPA to DHA. Furthermore, we found an increase in both HDHA and HEPE after treatment with DHA which reflects the increase of DHA content or conversion of DHA to EPA in HaCaT and 46BR.1N. The effect of PUFA on LOX products has also been studied in guinea pigs. Increased 15-HEPE was noted in animals fed with fish oil and increases in 15-HETrE was noted in animals fed with GLA (Miller et al., 1990). Another animal study shows increase EPA and DHA in epidermal phospholipids and their mediators, 15-HEPE and 17-HDHA, after supplementation with n-3 PUFA ethyl esters (Miller et al., 1991)

The results of this study show that both cells HaCaT keratinocytes and 46BR.1N fibroblasts can produce high levels of hydroxy fatty acids. However, 46BR.1N fibroblasts produce more of these than HaCaT keratinocytes do. Exposure of HaCaT and 46BR.1N to 15mJ/cm^2 UVR showed significant increases in HODE, HEPE, HETE and HDHA. Cells treated with EPA and DHA showed a significant decrease in the level of HODE and HETE mediators. Meanwhile, significant increases were found in HEPA and HDHA mediators post UVR and FA treatment. As a result, it seems that increased HODE and HETE may result from the effect of UVR on the LOX, while the increase in HEPE and HDHA may be a result not only of the effect of UVR on LOX, but also of the presence of their precursors (EPA and DHA respectively).

Chapter 8. Discussion and future work

8.1. Discussion

The human skin is a complex multilayered organ with several specialised appendages (Freinkel and Woodley, 2001). It is identified as the largest and one of the most important organs in the body (Bos, 2005), and it acts as a barrier between the body and external environment. The skin plays an important role in regulating body homeostasis by minimising water loss and regulating body temperature. Moreover, the nerve endings in the skin react to pain and temperature, and skin is an essential part of the immune system (Katunina, 2011). Skin condition and functioning are affected by environmental factors, such as UVR, free radicals, and toxic and allergic compounds, and by endogenous factors, such as genetic predisposition, immune and hormone status, and stress. Fatty acids and their derivates are considered important cellular structural and functional components. In the skin, fatty acids are important for the multilamellar lipid bilayer which regulates the permeability barrier and prevents excessive water loss and the entry of harmful substances into the skin (Proksck, 1999).

In this study, as shown in Table 5.1, the fatty acid profile of HaCaT epidermal keratinocytes (human cell line) and 46BR.1N dermal fibroblasts (human cell line) showed differences in their fatty acids composition; MUFA (54.22%) was the main group followed by SFA (37.11%), while OA (26.47%) was the main fatty acid followed by palmitic acid (18.96%) in HaCaT keratinocytes. Meanwhile, in 46BR.1N the SFA (51.94%) was the main group followed by MUFA (27.07%), and stearic acid (26.83%) was the main fatty acid followed by palmitic acid (20.68%). AA was the main n-6 PUFA and it was higher in 46BR.1N (8.40%) than in HaCaT (3.04%). Finally, DHA was found to

be the main n-3 PUFA and it was higher in 46BR.1N (2.68%) than in HaCaT (1.13%). These differences in the fatty acid composition of the epidermal and dermal cells could be quite useful for understanding the function and biochemistry of these histologically different regions of the skin (Uchida et al., 1988). However, more is known about the composition of fatty acids in human skin tissue and skin cell lines or about any changes in this composition during disease and cancer development (Mahadik et al., 1996).

We know that skin is the foremost organ protecting the body against environmental injuries, especially UVR in sun-light. It has been shown that UVR can damage biological macromolecules such as nucleic acids, lipids and proteins (Tsoyi et al., 2008). Furthermore, immune-suppression, carcinogenesis and apoptotic skin cell death are the result of chronic exposure to UVB. The inflammatory changes in response to acute UVB exposure on the skin include up-regulation of pro-inflammatory enzymes and their products. Numerous studies have shown that UVB irradiation significantly increased both mRNA and protein levels of COX-2 (Isoherranen et al., 1999, Rhodes et al., 2009, Lee et al., 2013b, Buckman et al., 1998). This in turn increases the production of pro-inflammatory mediators such as cytokines and PGs, specifically PGE_2 which has been reported to cause pain, oedema and vasodilatation in the inflamed regions (Mitchell et al., 1995, Siqueira et al., 2002).

Recently, attention has been focused on administering natural materials to protect skin against UV radiation. Diet has been recognised as a safe method of protecting the skin. Anti-inflammatory n-3 PUFAs have been suggested to have a beneficial role in keeping skin healthy and preventing disease. The purpose of this study was to measure and evaluate the protective effect of n-3

PUFAs, especially EPA and DHA, on skin cells against UVR, using a model system comprising HaCaT keratinocytes and 46BR.1N fibroblastic cells. Cells were treated with EPA, DHA and OA, and were exposed to UVR.

Competition with n-6 PUFA is one of the mechanisms behind the n-3 PUFA mediated protection. The result of this study showed that, treating HaCaT and 46BR.1N cells with EPA and DHA increased their cellular concentrations (chapter 5). More specifically, HaCaT cells treated with EPA resulted in significant increases of EPA, DPA and DHA, but AA was not affected by the EPA treatment. Increased EPA, DPA and DHA and decreased AA were observed when HaCaT cells were treated with DHA. Treating 46BR.1N cells with EPA resulted in significant increases of EPA, DPA and DHA but no significant decrease of AA. Increases of EPA, DPA and DHA and decrease of AA were observed when 46BR.1N cells were treated with DHA. Increased DPA and DHA after EPA treatment reflect its further metabolism by elongation enzymes. Unexpectedly, an increase of EPA and DPA content after DHA treatment not only indicates that DHA was incorporated into the cells, but also demonstrates the ability of these cells to retro-convert DHA to EPA and DPA. Conversion of DHA to EPA takes place in peroxisomes by the action of Δ4 enoyl reductase and Δ2, Δ3 enoyl CoA isomerise enzymes (Vidgren et al., 1997). In addition, an increase in the concentration of DPA after DHA treatment could be a result of saturation of DHA (Vidgren et al., 1997).

N-3 PUFA can alter signal transduction. When humans ingest up to 1.6 gm of EPA per day, a dose-related replacement of membrane PUFAs occurs. According to Flower and Perretti (2005), this result is achieved more effectively when the amount of AA in the diet is simultaneously reduced (Flower and

Perretti, 2005). Further to the above, when n-3 PUFAs are incorporated into membrane lipids, membrane composition is altered leading to increased viscosity and minor changes in osmotic cell weakness (Fischer and Black, 1991).

Here, the composition of fatty acids in HaCaT and 46BR.1N was studied only after EPA and DHA treatment and was not studied post UVR. The hypothesised that there would be a normal physiological replacement of fatty acids in the cells. Other studies have shown the effect of UVR on EPA and DHA levels in keratinocytes. Initially, the concentration of EPA and DHA in HaCaT cells was 0%. Following treatment with 50μM of fatty acid the content of EPA and DHA was increased to 3.4% and 6.3%, respectively, and then decreased to 2% and 5.4% after cells were exposed to 100mJ/cm^{2}UVB. (Storey et al., 2005). The concentration of EPA and DHA in CCD922SK fibroblasts increased from 5.7% and 3.2% in un-treated cells to 11.1% and 17.4% following EPA and DHA supplementation. This was then reduced to 10.3% and 14.8% after 100mJ/cm^2 UVB (Storey et al., 2005). We found that 50mJ/cm^2 decreased the viability of HaCaT and 46BR.1N to 50%, and this dose was considered toxic in our study.

UV irradiation had a negative effect on both the morphology of the cells and the growth parameters of the whole cell population. Many studies have reported that cell viability is reduced after exposure to UVR. The results of this study showed that HaCaT and 46BR.1N cell viability was decreased when these cells were exposed to various doses of UVR. Further decreases occurred when the cells were treated with EPA and DHA. On that basis, we hypothesised that this reduction in the cell viability may be due to induction of apoptosis. Apoptosis is

a controlled way used by tissues to remove abnormal cells. Deregulation of apoptosis is a hallmark of all cancer cells and can be considered a target for cancer therapeutics (Fadeel, 2004). PUFAs can regulate cellular proliferation and apoptosis through different mechanisms including gene expression, eicosanoid formation, signal transduction and lipid peroxidation (Field et al., 2002). Findings from this study show that there is an increase in cell apoptosis post UVR when the cells are treated with EPA and DHA. Long-chain n-3 PUFAs have a highly unsaturated structure. This property makes them the target of oxidation by free radicals, resulting in the formation of lipid peroxides. This lipid peroxidation is thought to be the mechanism for n-3 PUFA-induced apoptosis and prevention of the growth of tumour cells (Albino et al., 2000). A highly unsaturated structure of n-3 PUFA may explain our finding that, DHA which has more double bonds than EPA, induced more apoptosis than EPA. It has been reported that supplementation of humans with fish oil at 2g/day (18%EPA and 12%DHA) increased UV-induced production of lipid peroxidation. An increase in lipid peroxidation leads to a decrease in the ability of UV to induce erythema, which is partly mediated by ROS production. This suggests that the cellular structure is protected against oxidative damage by n-3 PUFA, which may act as oxidisable buffers (Rhodes et al., 1994). Moreover, apoptosis is also characterised by increased DNA fragmentation, decrease in nuclear size and translocation of the phosphatidyl serine phospholipid from inner to outer cell membrane. The ability of DHA to induce apoptosis to a greater extent than EPA may be a result of its ability to be incorporated in the phospholipid bilayer. The results show that HaCaT cells treated with 50 µM showed increased levels of DHA (7.20%) compared to EPA (3.02%) (Appendices 3.2 and 3.3). Moreover,

the level of DHA (15.06%) was greater than that of EPA (7.56%) in 46BR.1N after treatment with 50µM (Appendices 3.5 and 3.6). It has been reported that the effect of DHA in apoptosis could be due to its incorporation in the phospholipid bilayer, while EPA contributes to the formation of lipid bodies within the cell (Rudolph et al., 2001). Additionally, it was found that EPA and DHA induced the death of apoptotic cells in various cancer cell lines (Mund et al., 2007). This resulted in the increase of membrane unsaturation and fluidity with relocalisation of membrane signalling proteins followed by alteration in signal transduction and intracellular signalling pathways (Simopoulos, 2002). PUFAs have anti-carcinogenic properties facilitated via a reduced cancer cell growth, partly by cell signalling modification. Aspects of cellular development, growth and proliferation are mediated by the EGFR signalling pathway. Cancer development has been associated with mutations causing an increased expression or activation of EGFR. It has been reported that DHA supplementation consequently modulates EGFR phosphorylation, localisation and signalling. Further to the above, combining DHA with the EGFR inhibitor PD153035 was found to improve its actions (Rogers et al., 2010). Recently, it has been reported that both EPA and DHA can inhibit the growth of oral squamous cell carcinoma (SCC) cell lines but not normal keratinocytes. Low doses of EPA, in particular, inhibited the growth of pre-malignant and malignant keratinocytes by a combination of cell cycle arrest and apoptosis. Moreover, this was associated with the auto-phosphorylation of EGFR and the sustained phosphorylation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) (Nikolakopoulou et al., 2013).

N-3 PUFA can also act through an alteration of cytokine expression; the overproduction of pro-inflammatory cytokines is caused by an imbalance of n-6 and n-3 PUFAs. IL-1 and TNF are major proinflammatory cytokines. The impact of fish oil supplementation on the monocyte production of IL-1β and TNF-α was studied in nine healthy volunteers ingesting 16gm/day of fish oil. Six weeks after supplementation, reduction in IL-1β, IL- α and TNF-α cytokines was found (Endres, 1993). Furthermore, the long chain n-3 PUFAs, EPA and DHA decreased the baseline secretion of another pro-inflammatory mediator IL-8 and they decreased the post-UV release of IL-8 from keratinocytes and fibroblast cell lines. EPA and DHA also reduced the expected TNF-α stimulation of IL-8 secretion (Storey et al., 2005). In addition, studies have shown that n-3 PUFAs can modulate the expression of various genes as well as influencing the activation of numerous transcription factors. Changes in a cell's PUFA content influences a significant transcription factor such as NFkB. Increased regulation of NFkB is seen in response to stress and inflammation and has been known to activate numerous genes involved in carcinogenesis. Hardman (2002) reported that n-3 PUFAs were shown to prevent COX-2 gene expression by inhibition of NFkB (Hardman, 2002). The suppression of AA metabolism inhibited the activation of NFkB. Moreover, NFkB was found to be activated by PGE_2 and inhibited by EPA; this may indicate that possibility of a balancing role for an n-3: n-6 PUFA ratio in the important control of NFkB expression (Camandola et al., 1996). The peroxisome proliferator activated receptors (PPAR) are also affected by the n-3: n-6 PUFA ratio. These PPARs are nuclear receptors that regulate gene activity. PPAR-γ can be activated by metabolites of n-6 PUFA such as $PGE_{2,}$ which induce COX-2 expression. COX-2 expression can be deregulated

by the n-3 PUFA, EPA, in human keratinocytes via the PPAR-γ receptor. This indicates that a ratio of n-6: n-3 PUFA in the diet may regulate gene expression (Chene et al., 2007).

In order to measure the eicosanoids and related mediators produced by HaCaT and 46BR.1N cells, the cell culture media were analysed by LC/ESI-MS/MS. Results from HaCaT keratinocyte showed that the amount of prostaglandins, hydroxy fatty acids and epoxy fatty acids was 68, 109 and 4 pg/million cells (or 37.5%, 60.0% and 2.5% of total lipid extract) respectively. Analysis of 46BR.1N showed that the amount of prostaglandins, hydroxy fatty acids and epoxy fatty acids was 174, 614 and 8 pg/million cells (or 22%, 77.0% and 1% of total lipid extract) respectively. All mediators were found at higher levels in 46BR.1N than in HaCaT. The results also show that 13-HODE, PGE_2 and 12-HETE were the main mediators produced by HaCaT and 46BR.1N at baseline and post UVR. The level of these mediators decreased when the cells were treated with EPA and DHA, with or without UVR. PGE_3, 15-HEPE and 17-HDHA were the main n-3PUFA mediators. The levels of these mediators increased post UVR, and after EPA and DHA treatment. It has been known that PGE_2 has an important role in keratinocyte proliferation and apoptosis during homeostasis (Chun et al., 2007, Chun et al., 2010). Furthermore, PGE_2 had been mediating the vasodilatation of UVR inducing an inflammatory response in humans. 12-HETE has chemoattractant properties that have been important in some skin diseases such as psoriasis (Barr et al., 1984) and inflamed skin (Black et al., 1985). The actions of anti-inflammatory mediators in the skin are not completely known, but increasing the level of anti-inflammatory mediators and inducing a significant reduction in inflammatory mediators following UVR

exposure, suggests that increased content of EPA and DHA may contribute to the regulation of cutaneous inflammatory responses. The UVR increases the liberation of AA and other PUFAs from membrane phospholipids. EPA competes with AA, and although it is no better than AA substrate for COX, EPA produces $PGE_{3,}$ which is markedly less effective than PGE_2. The results of this study, as expected, showed a potentially protective effect of n-3 PUFAs against UVR, but the study was not expected to find that DHA had a more potent role than EPA. Specifically: DHA decreases cell viability, increases cell apoptosis and decreases pro-inflammatory mediators such as PGE_2 post UVR more than EPA.

Overall, this study suggests that the protective role of n-3 PUFAs may be the result of inhibiting the activity of COX and LOX. Our results show that the epoxy fatty acid 14,(15)-EET was detected in HaCaT and 46BR.1N cells. Furthermore, the dihydroxy fatty acid 14, 15-DHET was detected in HaCaT and 46BR.1N cells. The levels of these mediators were increased post UVR and were not affected by the n-3 PUFA treatment.

As a conclusion from the above, the *in vitro* experiments suggest that n-3 PUFAs have a beneficial role when incorporated in cells and increase their concentration. This result in increased cell death post UVR, decreased inflammatory mediators such as PGE_2 and increased anti-inflammatory mediators such as PEG_3, HEPE and HDHA. The next question should be as follows: Do the n-3 PUFAs have the same effect *in vivo*? In order to answer this question a clinical study was performed using healthy volunteers. The active group was supplemented with 4g/day n-3 PUFAs. The content of fatty acid in RBC was analysed to show the compliance, and skin (dermal) fatty acid was

used to assess the target system. The content of EPA, DPA and DAH in RBC increased after n-3 PUFA supplementation while only EPA in dermal tissue was significantly increased after n-3PUFA supplementation. Although the aim of this study was achieved when by monitored the increase in the content of EPA in the skin, but there was no big change in the amount of EPA. This may because the baseline EPA was lower in this group of volunteers (Wallingford et al., 2012).

8.2. Future work

Many of the results of this study were in agreement with other studies in that a decrease in cell viability was observed when cells were treated with n-3 PUFAs and exposed to UVR. The results also suggest that both UVR and the n-3 PUFA EPA and DHA increase apoptosis in HaCaT and 46BR.1N cells in a dose-dependent manner. Moreover, further increases in apoptosis were observed when cells were treated with n-3 PUFAs and exposed to UVR. More studies are needed to fully understand the mechanism of this action and to assess the effect of UVR and n-3 PUFAs in the extrinsic and intrinsic pathways of apoptosis.

Caspases are central components for apoptosis. Caspases involved in apoptosis are generally divided into two groups, the initiator caspases, which include caspase-2, -8, -9, and -10, and the effector caspases, which include caspase-3, -6, and -7. It will be useful to assess the capacity of n-3 PUFA to activate the caspases. This can be achieved by treating the HaCaT keratinocytes and 46BR.1N fibroblasts cells with and without n-3 PUFA in the presence and absence of specific inhibitors of the different caspases. Changes in levels of caspase expression can be measured using western blotting and changes in activity by specific ELISAs. These experiments will clarify which

caspases important in apoptosis are modulated by n-3 PUFA. Also in parallel with this, it will be useful to compare other compounds which play an important role in modulating the apoptosis pathways such as cytochrome *c*, BAX and tBid. A better understanding of how n3 PUFA modulates apoptosis may help to explain the mechanisms of action for these fatty acids in the skin.

Activation of the extrinsic apoptosis pathway may also be a mechanism of n-3 PUFA action to induce apoptosis. This pathway is commonly activated by death receptors. Death receptors may be activated directly by the fatty acids or their bioactive mediators. This could be assessed by treating the HaCaT keratinocytes and 46BR.1N fibroblasts cells with n-3 PUFA in the presence and absence of specific inhibitors of the death receptors.

The intracellular concentration of ROS appears to dictate stress survival apoptotic responses. Low levels of ROS are utilised within the cells as signalling intermediates for normal homeostasis. However, high levels of ROS directly damages cellular structural components and can induce apoptosis. Furthermore, it has been reported that n-3 PUFA produces ROS resulting in the formation of lipid peroxides. According to this, production of ROS can be a mechanism for n-3 PUFA-induced apoptosis and prevention of the growth of cancer cells. This mechanism can be followed by treating the HaCaT keratinocyte and 46BR.1N fibroblasts cells with or without n-3 PUFA and with or without a ROS inhibiter such as diphenyleneiodoniuma and N-acetyl-L-cysteine.

Analysis of fatty acid profile on HaCaT and 46BR.1N with and without n-3 PUFA treatment showed an increase in the content of these fatty acids in the cells. It may be better to analyse the fatty acid profile in these cells post

UVR with and without n-3 PUFA treatment. This may help to explain and provide an understanding of the effect of UVR on the fatty acid profile of these cells, PUFAs in prostanoids and hydroxy fatty acids.

The results in this study showed an increase in inflammatory mediators such as PGE_2 post UVR and showed that n-3PUFA reduced pro-inflammatory and increased anti-inflammatory mediators such as PGE_3. To fully understand these findings, we must explore their mechanism of action so that we can understand how they work. Further studies are needed to investigate the relationship between n-3 PUFA mediators and gene expression and protein expression of lipid enzymes such as COX and LOX. All the experiments in this study were performed at 24h post UVR. It would be beneficial to repeat these experiments with more time points before and after 24h to investigate the up or down-regulation of COX and LOX expression and their bioactive lipids.

Studies with EPA and DHA suggest that their effect of protecting the skin against UVR may be related to their direct action, or to the action of their metabolites. More experiments are needed to investigate which of these fatty acids performs best in protecting skin and treating skin disorders. Moreover, the results showed an increase in the level of DPA post EPA and DHA treatment. More studies are needed to investigate the specific biological properties and active biological mediators of DPA. It will be more useful to treat the HaCaT keratinocytes and 46BR.1N with DPA to investigate; A) The effect of DPA on HaCaT and 46BR.1N cells viability and apoptosis, B) The effect of DPA on the eicosanoids produced by HaCaT and 46BR.1N cells, C) The effect of DPA on COX and LOX expression. This can be achieved by treating the cells with DPA and then comparing the viability and apoptosis of cells that have been exposed

to UVR with those that have not. Also the effects of DPA on the production of eicosanoids can be assessed by analysis of the cell medium by LC/ESI-MS/MS.

Finally, all in vitro experiments in this study were performed on HaCaT and 46BR.1N cells which are cell lines. It would be more beneficial to the understanding of the effect of n-3PUFA on skin cells to repeat these experiments on primary human skin cells such as primary keratinocytes and primary fibroblasts. Skin punch biopsies should take from a site that has not been exposed to UVR, to avoid any cellular changes induced by UVR. Moreover, the clinical study was performed in order to assess the effect of n-3 PUFA on skin fatty acids profile. After three months of EPA ethyl ester (~72%) and DHA ethyl ester (10%) 4g daily supplementation only the composition of EPA was slightly increased in skin post supplementation. It will be useful to design and apply a new clinical study which increases the amount of DHA in the supplementation and the daily dose. This will increase the composition of n-3 PUFA in skin which has a role of protecting it against UVR.

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Appendices

APPENDIX.1

APPENDIX 1.1

Complete RPMI 1640 medium

RPMI 1640 media 450ml

Fetal calf serum 50ml

Penicillin (10,000U/ml) Streptomycin (10mg/ml)

Amphotericin B (2.5ng/ml)

RPMI 1640 serum free medium

RPMI 1640 media 500ml

Penicillin (10,000U/ml) Streptomycin (10mg/ml)

Amphotericin B (2.5ng/ml)

Complete Dulbecco's Modified Eagle Medium (DMEM)

DMEM media 450ml

Fetal calf serum 50ml

Sodium Pyruvate 1mM

Penicillin (10,000U/ml) Streptomycin (10mg/ml)

Amphotericin B (2.5ng/ml)

Dulbecco's Modified Eagle (DMEM) serum free Medium

DMEM media 500ml

Sodium Pyruvate 1mM

Penicillin (10,000U/ml) Streptomycin (10mg/ml) Amphotericin B (2.5ng/ml)

Complete Minimum Essential Medium Eagle (MEME)

MEME media 425ml

L-glutamine 2mM

Non Essential Amino acids 1%

Sodium Pyruvate 1mM

Fetal Bovine serum 75ml

Penicillin (10,000U/ml) Streptomycin (10mg/ml)

Amphotericin B (2.5ng/ml)

Complete Minimum Essential Medium Eagle (MEME) serum free Medium

MEME media 500ml

L-glutamine 2mM

Non Essential Amino acids 1%

Sodium Pyruvate 1mM

Penicillin (10,000U/ml) Streptomycin (10mg/ml)

Amphotericin B (2.5ng/ml)

Oleic Acid stock solution (50mM)

Oleic acid 26 mg

Dimethyl sulfoxide (DMSO) 1.84 ml

Aliquoted (55µl each) and stored at -20°C. Each aliquot is used only once.

Eicosapentaenoic Acid stock solution (50mM)

Eicosapentaenoic Acid 10 mg

Dimethyl sulfoxide (DMSO) 0.66 ml

Aliquoted (55µl each) and stored at -20°C. Each aliquot is used only once.

Docosahexaenoic Acid stock solution (50mM)

Docosahexaenoic acid 25 mg

Dimethyl sulfoxide (DMSO) 1.5 ml

Aliquoted (55µl each) and stored at -20°C. Each aliquot is used only once.

10mM H_2O_2

100µl H_2O_2 1M

900 µl media

5%V/V Dye

500µl APOPercentage Dye

9500 µl cell culture media

Reagent A and B

	Reagent A 500 µl/well	**Reagent B 500 µl/well**
Reagent Blank	Culture media	Reagent A
Negative control	Culture media	Reagent A + 5%V/V Dye
Positive control	Culture media+ reference apoptotic agent	Reagent A + 5%V/V Dye
Test samples	Culture media + test apoptotic agent	Reagent A + 5%V/V Dye

APPENDIX 1.2

0.01% (w/v) BHT in 2:1 chloroform: methanol

0.030 g BHT dissolved in 200ml chloroform and 100ml methanol and stored at room temperature for a month.

0.5M Potassium chloride (KCL) in 50% methanol

7.455 g potassium chloride dissolved in 100ml water and 100ml methanol and stored at room temperature for a month

Toluene: Methanol (50/50) (v/v)

10 ml toluene mixed with 10 ml methanol. Stored in a clean glass container at 4°C.

10% (w/v) Potassium Carbonate (K_2CO_3) solution:

10.0 g potassium carbonate dissolved in 100ml deionised water and stored at room temperature for a month.

APPENDIX 1.3

Hydrochloric acid (HCl) 1M

8.5 ml HCl

100ml water

Methanol 15%

45ml methanol

255ml water

APPENDIX. 2

Appendix 2.1 Fatty acid profile of RBC pre and post n-3PUFA supplementation in Placebo and Active groups. Results are expressed as % weight of total fatty acid (mean ± SEM).

FATTY ACID	Placebo PRE n=33	Placebo POST n=33	Active PRE n=35	Active POST n=35
C12:0	0.08 ± 0.01	0.10 ± 0.02	0.11± 0.02	0.10 ±0.02
C14:0	0.62 ± 0.11	0.63 ±0.11	0.70 ± 0.12	0.63 ± 0.11
C15:0	0.16 ± 0.03	0.15 ±0.03	0.15 ± 0.03	0.15 ± 0.03
C16:0	26.19 ± 4.56	25.18 ± 4.38	24.93 ± 4.15	24.44 ± 4.13
C17:0	0.31 ± 0.05	0.28 ± 0.05	0.32 ± 0.05	0.31± 0.05
C18:0	15.30 ± 2.66	14.81± 2.58	14.51 ± 2.42	14.41± 2.44
C20:0	0.30 ± 0.05	0.25 ± 0.04	0.247 ± 0.04	0.23± 0.04
C22:0	0.62 ± 0.11	0.48 ± 0.08	0.57 ± 0.09	0.54 ± 0.09
C23:0	0.10 ± 0.02	0.07 ± 0.01	0.0744 ± 0.01	0.08 ± 0.01
C24:0	1.71 ± 0.30	1.46 ± 0.25	1.6672 ± 0.28	1.36 ± 0.23
ΣSFA	**45.38 ± 7.90**	**43.41 ± 7.56**	**43.25 ± 7.21**	**42.26 ± 7.14**
C14:1	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
C15:1	0.01 ± 0.01	0.01 ±0.01	0.01 ± 0.01	0.01 ± 0.01
C16:1	0.80 ± 0.14	1.05 ±0.18	0.87 ± 0.15	0.71 ±0.12
C17:1	0.01 ±0.01	0.01 ± 0.01	0.01 ± 0.01	0.03 ±0.01
C18:1n-9t	0.12 ±0.02	0.09 ± 0.02	0.14 ± 0.02	0.06 ±0.01
C18:1n-9c	16.58 ±2.89	16.57 ±2.88	16.67 ± 2.78	16.67 ± 2.82
C18:1n-7	1.19 ±0.21	1.13 ±0.20	1.12 ± 0.19	1.44 ± 0.24
C20:1n-9	0.37 ±0.06	0.45 ±0.08	0.54 ± 0.09	0.31 ± 0.05
C22:1n-9	0.10 ± 0.02	0.09 ±.0.02	0.09 ± 0.01	0.11±0.02
C24:1	1.73 ±.30	1.40 ±.0.24	1.49 ± 0.25	1.49 ± 0.25
ΣMUFA	**20.93 ± 3.64**	**20.79 ± 3.62**	**20.94 ± 3.49**	**20.84 ± 3.52**
C18:2n-6t	0.06 ± 0.01	0.06±0.01	0.06 ± 0.01	0.03 ± 0.01
C18:2n-6c	15.05 ± 2.62	15.51± 2.70	14.23 ± 2.37	13.73 ± 2.32
C18:3n-6	0.17 ± 0.03	0.16 ±.0.03	0.21 ± 0.03	0.11 ± 0.02
C20:3n-6	1.96 ± 0.34	1.97 ± 0.34	1.80 ± 0.30	1.56 ± 0.26
C20:4n-6	13.45 ± 2.34	12.77 ± 2.22	12.31 ± 2.05	11.84 ± 2.00
ΣN-6PUFA	**30.69 ± 5.34**	**30.46 ± 5.30**	**28.61 ± 4.77**	**27.28 ± 4.61**
C18:3n-3	0.34 ±.0.06	0.37 ±.0.07	0.46 ±.0.08	0.31 ± 0.05
C20:3n-3	0.01 ± 0.01	0.03±.0.01	0.02 ±0.001	0.02 ± 0.001
C20:5n-3	0.90 ± 0.16	0.92 ±0.16	0.93 ±.0.15	3.61 ± 0.61
C22:5n-3	1.98 ± 0.35	1.93 ±.0.34	1.85 ±0.3	3.19 ± 0.54
C22:6n-3	3.53 ±.0.62	3.53 ±0.61	3.61 ±0.60	4.20 ± 0.71
ΣN-3PUFA	**6.76 ± 1.18**	**6.79 ± 1.18**	**6.88 ± 1.15**	**11.34 ±1.92**
C20:2	0.18 ± 0.03	0.26 ± 0.05	0.19 ±0.03	0.20 ±0.03
C22:2	0.06 ±0.01	0.04 ± 0.01	0.07 ±0.01	0.06 ±0.01

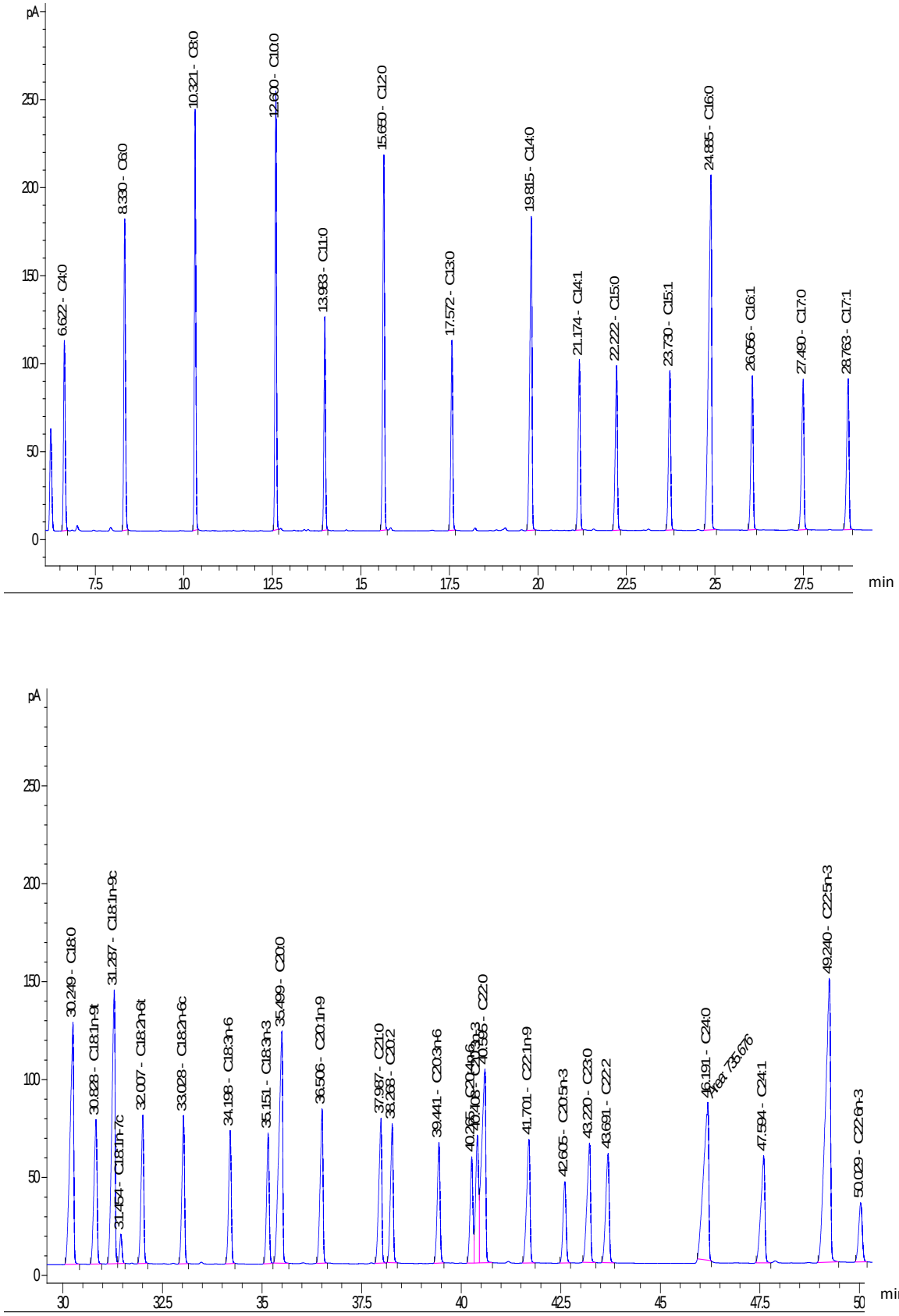
Appendix 2.2 Fatty acid profile of dermis pre and post supplementation, in the Placebo and Active groups. Results are expressed as % weight of total fatty acid (mean ± SEM).

FATTY ACID	Placebo PRE n=14	Placebo POST n=14	Active PRE n=19	Active POST n=19
C12:0	0.18 ± 0.05	0.21 ± 0.05	0.18 ± 0.04	0.17 ± 0.04
C14:0	1.82 ± 0.49	1.82 ± 0.49	1.84 ± 0.42	1.76 ± 0.40
C15:0	0.22 ± 0.06	0.24 ± 0.06	0.24 ± 0.05	0.21 ± 0.05
C16:0	19.97 ± 5.34	20.52 ± 5.48	21.85 ± 5.01	19.06 ± 4.37
C17:0	0.19 ± 0.05	0.23 ± 0.06	0.21 ± 0.05	0.13 ± 0.03
C18:0	2.75 ± 0.73	3.72 ± 0.99	3.66 ± 0.84	2.57 ± 0.59
C20:0	0.16 ± 0.04	0.13 ± 0.03	0.20 ± 0.05	0.16 ± 0.04
C22:0	0.02 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.01 ±0.01
C23:0	0.01 ± 0.01	0.01 ± 0.01	0.03 ± 0.01	0.00 ±0.00
C24:0	0.07 ± 0.02	0.07 ± 0.02	0.06 ± 0.01	0.05 ± 0.01
ΣSFA	**25.38 ± 6.78**	**26.97 ± 7.71**	**28.31 ± 6.50**	**24.13 ± 5.54**
C14:1	0.52 ± 0.14	0.49 ± 0.13	0.52 ± 0.12	0.57 ± 0.13
C15:1	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01
C16:1	9.76 ± 2.61	8.21 ± 2.19	10.13 ± 2.23	10.38 ± 2.38
C17:1	0.17 ± 0.05	0.17 ± 0.04	0.19 ± 0.04	0.21 ± 0.05
C18:1n-9t	0.92 ± 0.25	0.72 ± 0.19	0.80 ± 0.18	0.34 ± 0.08
C18:1n-9c	45.81 ± 12.24	46.01 ±12.30	42.28 ± 9.70	46.82 ± 10.74
C18:1n-7	2.93 ± 0.78	3.28 ± 0.88	3.20 ± 0.73	2.75 ± 0.63
C20:1n-9	0.45 ± 0.12	0.44 ±0.12	0.37 ± 0.08	0.39 ± 0.09
C22:1n-9	0.05 ± 0.01	0.06 ± 0.02	0.07 ± 0.02	0.03 ± 0.01
C24:1	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.02 ± 0.01
ΣMUFA	**60.66 ± 16.21**	**59.43 ± 15.88**	**57.61 ± 13.22**	**61.52 ± 14.11**
C18:2n-6t	0.14 ±0.04	0.14 ± 0.04	0.14 ± 0.03	0.10 ± 0.02
C18:2n-6c	11.04 ± 3.07	10.99 ± 2.94	10.58 ± 2.43	11.63 ± 2.67
C18:3n-6	0.07 ± 0.02	0.08 ± 0.02	0.06 ± 0.01	0.07 ± 0.02
C20:3n-6	0.21 ± 0.06	0.22 ± 0.06	0.22 ± 0.05	0.19 ± 0.04
C20:4n-6	0.57± 0.15	0.66 ± 0.18	0.73 ± 0.17	0.61 ± 0.14
ΣN-6PUFA	**12.47** ± 3.33	**12.08 ± 3.23**	**11.74** ± 2.69	**12.60 ± 2.89**
C18:3n-3	0.76 ± 0.20	0.69 ± 0.19	0.74 ± 0.17	0.81 ± 0.19
C20:3n-3	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
C20:5n-3	0.05 ± 0.01	0.06 ± 0.02	0.08 ± 0.02	0.12 ± 0.03 [a,b]
C22:5n-3	0.18 ± 0.05	0.19 ±0.05	0.21 ± 0.05	0.24 ± 0.05
C22:6n-3	0.14 ± 0.04	0.16 ± 0.04	0.20 ± 0.05	0.21 ± 0.05
ΣN-3PUFA	**1.17 ± 0.31**	**1.14 ±0.31**	**1.27 ±0.29**	**1.42 ±0.33**
C20:2	0.31 ± 0.08	0.36 ± 0.10	1.05 ± 0.24	0.32 ± 0.07
C22:2	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01

Appendix 2.3 Fatty acid profile of human epidermis, dermis and RBC. Results are expressed as % weight of total fatty acid (mean ± SD)

Fatty acid	Epidermis n=5	Dermis n=5	RBC n=5
C12:0	0.43 ± 0.20	0.18 ± 0.05	0.06 ± 0.06
C14:0	3.03 ±1.24	1.86 ± 0.43	0.62 ± 0.13
C15:0	0.96 ± 0.73	0.25 ± 0.05	0.15 ± 0.04
C16:0	20.97 ±1.61	19.73 ± 6.69	24.81 ± 1.01
C17:0	0.50 ± 0.05	0.16 ± 0.12	0.31 ± 0.08
C18:0	10.65 ± 0.76	2.83 ± 1.35	13.52 ± 0.90
C20:0	0.28 ± 0.06	0.17 ± 0.09	0.30 ± 0.09
C22:0	0.00 ± 0.00	0.02 ± 0.01	0.68 ± 0.15
C23:0	0.15 ± 0.01	0.01 ± 0.01	0.11 ± 0.03
C24:0	0.34 ± 0.12	0.03 ± 0.02	1.50 ± 0.53
ΣSFA	**37.29 ± 4.52**	**25.24 ± 8.03**	**42.08 ± 1.11**
C14:1	0.31 ± 0.12	0.68 ± 0.35	0.01 ± 0.01
C15:1	0.00 ± 0.00	0.05 ± 0.03	0.01 ± 0.01
C16:1	6.36 ± 1.28	11.38 ± 3.66	0.99 ± 0.53
C17:1	0.30 ± 0.01	0.23 ± 0.15	0.01 ± 0.03
C18:1n-9t	0.17± 0.02	0.40 ± 0.25	0.03 ±0.02
C18:1n-9c	35.09 ± 3.25	44.93 ± 7.53	17.58 ± 1.49
C18:1n-7	2.73 ± 0.56	3.22 ± 0.69	1.27 ± 0.22
C20:1n-9	0.43 ± 0.08	0.41 ± 0.05	0.25 ± 0.03
C22:1n-9	0.35 ± 0.06	0.04 ± 0.02	0.08 ± 0.04
C24:1	0.20 ± 0.07	0.03 ± 0.02	2.28 ± 0.69
ΣMUFA	**45.94 ± 4.99**	**61.36 ± 8.61**	**22.50 ± 2.04**
C18:2n-6t	0.45 ± 0.33	0.11 ± 0.07	0.02 ± 0.01
C18:2n-6c	10.77± 0.62	10.85 ± 1.94	14.28 ± 3.38
C18:3n-6	0.11 ± 0.05	0.09 ± 0.001	0.16 ± 0.03
C20:3n-6	0.49 ± 0.16	0.18 ± 0.06	1.92 ± 0.50
C20:4n-6	2.32 ± 0.23	0.69 ± 0.14	13.51 ± 1.32
ΣN-6PUFA	**14.13 ± 0.67**	**11.92 ± 1.98**	**29.89 ± 2.47**
C18:3n-3	0.47± 0.09	0.73 ± 0.29	0.39 ± 0.11
C20:3n-3	0.42 ± 0.01	0.03 ± 0.01	0.00 ± 0.00
C20:5n-3	0.61± 0.94	0.07 ± 0.02	0.77 ± 0.47
C22:5n-3	0.41± 0.03	0.18 ± 0.05	1.78 ± 0.17
C22:6n-3	0.38 ± 0.08	0.19 ± 0.07	3.83 ± 0.51
ΣN-3PUFA	**2.29 ± 1.00**	**1.20 ± 0.30**	**6.77 ± 0.93**
C20:2	0.16 ± 0.04	0.27 ± 0.25	0.26 ± 0.24
C22:2	0.18 ±0.16	0.01 ± 0.01	0.06 ± 0.01

Appendix 2.4 Sample GC-FID chromatogram of cocktail FAME standard (SIGMA).



APPENDIX .3

Appendix.3.1. Fatty acid profile of HaCaT cells before (control) and after treatment with OA 10µM and 50µM. Results are expressed as % weight of total fatty acids (mean ± SD) of n=3 independent experiments.

FATTY ACID	Control	OA 10M	OA 50M
C12:0	0.05 ± 0.04	0.11 ± 0.06	0.15 ± 0.06
C14:0	1.52 ± 0.32	1.71 ± 0.22	1.82 ± 0.26
C15:0	0.11 ± 0.07	0.14 ± 0.04	0.15 ± 0.04
C16:0	18.96 ± 2.48	17.58 ± 1.22	18.29 ± 1.52
C17:0	0.26 ± 0.12	0.16 ± 0.05	0.19 ± 0.07
C18:0	15.32 ± 8.09	9.19 ± 2.94	9.99 ± 3.76
C20:0	0.23 ± 0.08	0.27 ± 0.09	0.29 ± 0.10
C22:0	1.0 ± 001	0.00 ± 00.	0.00 ± 00
C23:0	0.03 ± 0.01	0.04 ± 0.001	0.04 ± 0.02
C24:0	0.64 ± 0.53	0.42 ± 0.30	0.41 ± 0.28
ΣSFA	**37.11 ± 9.16**	**29.62 ± 4.93**	**31.31 ± 6.11**
C14:1	0.03 ± 0.10	0.16 ± 0.08	0.15 ± 0.11
C16:1	9.85 ± 4.90	10.90 ± 3.88	10.44 ± 3.85
C17:1	0.53 ± 0.08	0.46 ± 0.34	0.39 ± 0.26
C18:1n-9t	0.09 ± 0.03	0.14 ± 0.03	0.32 ± 0.27
C18:1n-9c	26.47 ± 1.77	31.53 ± 5.00	31.55 ± 4.11
C18:1n-7	14.85 ± 5.17	16.42 ± 3.77	15.75 ± 4.26
C20:1n-9	1.51 ± 0.39	1.96 ± 0.16	1.97 ± 0.16
C22:1n-9	0.48 ± 0.32	0.69 ± 0.06	0.73 ± 0.34
C24:1	0.35 ± 0.24	0.83 ± 0.68	0.47 ± 0.36
ΣMUFA	**54.22 ± 8.82**	**63.10 ± 14.01**	**61.76 ± 13.73**
C18:2n-6t	0.10 ± 0.09	0.16 ± 0.22	0.05 ± 0.02
C18:2n-6c	2.19 ± 1.00	1.61 ± 0.49	1.56 ± 0.43
C18:3n-6	0.13 ± 0.25	0.06 ± 0.05	0.06 ± 0.05
C20:3n-6	0.43 ± 0.15	0.55 ± 0.34	0.40 ± 0.08
C20:4n-6	3.04 ± 1.69	2.25 ± 0.80	2.08 ± 0.58
ΣN-6PUFA	**5.88 ± 2.64**	**4.62 ± 1.89**	**4.15 ± 1.15**
C18:3n-3	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
C20:3n-3	0.33 ± 0.08	0.37 ± 0.14	0.42 ± 0.11
C20:5n-3	0.25 ± 0.09	0.33 ± 0.07	0.32 ± 0.07
C22:5n-3	1.00 ± 0.26	0.94 ± 0.11	0.97 ± 0.19
C22:6n-3	1.13 ± 0.23	1.09 ± 0.12	1.09 ± 0.28
ΣN-3PUFA	**2.72 ± 0.49**	**2.75 ± 0.45**	**2.83 ± 0.66**
C20:2	0.18 ± 0.09	0.13 ± 0.06	0.14 ± 0.05
C22:2	0.04 ± 0.02	0.02 ± 0.01	0.07 ± 0.06

Appendix.3.2. Fatty acid profile of HaCaT cells before (control) and after treatment with EPA 10µM and 50µM. Results are expressed as % weight of total fatty acid (mean ± SD) of n=3 independent experiments. (a) comparing control to EPA10µM group and (b) comparing control to EPA 50µM group. ^{a,b}=p≤0.05, ^{aa,bb}=p≤0.001,^{aaa,bbb}=p≤0.001.

FATTY ACID	Control	EPA 10M	EPA 50M
C12:0	0.05 ± 0.04	0.11 ± 0.01	0.12 ± 0.07
C14:0	1.52 ± 0.32	2.13 ± 0.33	1.97 ± 0.35
C15:0	0.11 ± 0.07	0.21 ± 0.05	0.21 ± 0.06
C16:0	18.96 ± 2.48	19.34 ± 0.64	19.20 ± 1.30
C17:0	0.26 ± 0.12	0.19 ± 0.03	0.19 ± 0.03
C18:0	15.32 ± 8.09	9.67± 1.21	10.95 ± 1.30
C20:0	0.23 ± 0.08	0.21± 0.04	0.22 ± 0.02
C22:0	0.0 001	0000 ± 000	0.00 ± 0.001
C23:0	0.03 ± 0.01	0.03 ± 0.02	0.03 ± 0.001
C24:0	0.64 ± 0.53	0.96 ± 0.26	0.72 ± 0.16
ΣSFA	**37.11 ± 9.16**	**32.85 ± 2.59**	**33.62 ± 3.29**
C14:1	0.03 ± 0.10	0.17 ± 0.09	0.14 ± 0.02
C16:1	9.85 ± 4.90	11.39 ± 2.64	10.47 ± 1.44
C17:1	0.53 ± 0.08	0.43 ± 0.38	0.48 ± 0.42
C18:1n-9t	0.09 ± 0.03	0.08 ± 0.01	0.08 ± 0.02
C18:1n-9c	26.47 ± 1.77	25.65 ± 0.87	24.63 ± 1.82
C18:1n-7	14.85 ± 5.17	14.48 ± 3.08	10.09 ± 1.89
C20:1n-9	1.51 ± 0.39	1.31 ± 0.46	0.83 ± 0.33
C22:1n-9	0.48 ± 0.32	0.32 ± 0.29	0.26 ± 0.28
C24:1	0.35 ± 0.24	0.19 ± 0.01	0.16 ± 0.02
ΣMUFA	**54.22 ± 8.82**	**54.02 ± 7.83**	**47.14 ± 6.23**
C18:2n-6t	0.10 ± 0.09	0.09 ± 0.03	0.09 ± 0.03
C18:2n-6c	2.19 ± 1.00	2.79 ± 0.84	2.84 ± 0.76
C18:3n-6	0.13 ± 0.25	0.08 ± 0.07	0.14 ± 0.02
C20:3n-6	0.43 ± 0.15	0.67 ± 0.24	1.86 ± 0.34
C20:4n-6	3.04 ± 1.69	4.44 ± 1.42	3.07 ± 1.19
ΣN-6PUFA	**5.88 ± 2.64**	**8.07 ± 2.60**	**8.09 ± 2.33**
C18:3n-3	0.03 ± 0.01	0.04 ± 0.02	0.13 ± 0.08
C20:3n-3	0.33 ± 0.08	0.34 ± 0.08	0.38 ± 0.02
C20:5n-3	0.25 ± 0.09	0.65 ± 0.07	3.02 ± 0.44^{bbb}
C22:5n-3	1.00 ± 0.26	2.62 ± 0.63^{aa}	6.00 ± 1.18^{bbb}
C22:6n-3	1.13 ± 0.23	1.24 ± 0.36	1.48 ± 0.26
ΣN-3PUFA	**2.72 ± 0.49**	**4.89 ± 1.16**	**11.02 ± 1.98^{bbb}**
C20:2	0.18 ± 0.09	0.22 ± 0.04	0.16 ± 0.01
C22:2	0.04 ± 0.02	0.03 ± 0.00	0.02 ± 0.001

Appendix.3.3. Fatty acid profile of HaCaT cells before (control) and after treatment with DHA 10μM and 50μM. Results are expressed as % weight of total fatty acid (mean ± SD) of n=3 independent experiments. (a) comparing control to DHA10μM group and (b) comparing control to DHA50μM group. [a,b]=p≤0.05, [aa,bb]=p≤0.001.

FATTY ACID	Control	DHA 10M	DHA 50M
C12:0	0.05 ± 0.04	0.15 ±0.12	0.08 ± 0.04
C14:0	1.52 ± 0.32	1.60 ±0.10	1.28 ± 0.12
C15:0	0.11 ± 0.07	0.15 ±0.03	0.15 ± 0.03
C16:0	18.96 ± 2.48	17.02 ±2.50	16.85 ± 1.10
C17:0	0.26 ± 0.12	0.19 ±0.08	0.23 ± 0.06
C18:0	15.32 ± 8.09	10.45 ±4.28	13.03 ± 2.49
C20:0	0.23 ± 0.08	0.24 ±012	0.31 ± 0.07
C22:0	1.0 ± 001	0.01 ±001	0.00 ± 0.001
C23:0	0.03 ± 0.01	0.03 ±0.01	0.01 ± 0.05
C24:0	0.64 ± 0.53	0.40 ±0.28	0.28 ± 0.21
ΣSFA	**37.11 ± 9.16**	**30.22 ±7.53**	**32.29 ± 4.18**
C14:1	0.03 ± 0.10	0.17 ±0.13	0.09 ± 0.05
C16:1	9.85 ± 4.90	10.96 ±4.76	8.37 ± 2.43
C17:1	0.53 ± 0.08	0.32 ±0.28	0.36 ± 0.24
C18:1n-9t	0.09 ± 0.03	0.13 ±0.05	0.12 ± 0.05
C18:1n-9c	26.47 ± 1.77	30.19 ±4.43	31.74 ± 3.09
C18:1n-7	14.85 ± 5.17	15.74 ±5.33	9.86 ± 2.71
C20:1n-9	1.51 ± 0.39	1.88 ±0.28	1.21 ± 0.44
C22:1n-9	0.48 ± 0.32	0.64 ±0.06	0.60 ± 0.14
C24:1	0.35 ± 0.24	0.47 ±0.26	0.51 ± 0.46
ΣMUFA	**54.22 ± 8.82**	**60.51 ±15.59**	**52.87 ± 0.61**
C18:2n-6t	0.10 ± 0.09	0.19 ±0.24	0.17 ± 0.18
C18:2n-6c	2.19 ± 1.00	2.16 ±1.26	2.21 ± 1.05
C18:3n-6	0.13 ± 0.25	0.03 ±0.01	0.07 ± 0.09
C20:3n-6	0.43 ± 0.15	0.47 ±0.09	0.79 ± 0.14
C20:4n-6	3.04 ± 1.69	2.51 ±1.18	2.44 ± 1.06
ΣN-6PUFA	**5.88 ± 2.64**	**5.36 ±2.78**	**5.68 ± 2.52**
C18:3n-3	0.03 ± 0.01	0.03 ±0.01	0.08 ± 0.05
C20:3n-3	0.33 ± 0.08	0.33 ±0.14	0.54 ± 0.20
C20:5n-3	0.25 ± 0.09	0.38 ±0.18	1.36 ± 1.11[b]
C22:5n-3	1.00 ± 0.26	1.10 ±0.09	1.46 ± 0.34
C22:6n-3	1.13 ± 0.23	2.08 ±0.97	7.20 ± 3.26[bb]
ΣN-3PUFA	**2.72 ± 0.49**	**3.93 ±1.39**	**10.63 ± 4.96[bb]**
C20:2	0.18 ± 0.09	0.19 ±0.11	0.16 ± 0.09
C22:2	0.04 ± 0.02	0.05 ±0.05	0.03 ± 0.02

Appendix.3.4. Fatty acid profile of 46BR.1Ncells before (control) and after treatment with OA 10µM and 50µM. Results are expressed as % weight of total fatty acid (mean ± SD) of n=3 independent experiments. (a) comparing control to OA10µM group and (b) comparing control to OA 50µM group. [a,b]=p≤0.05.

FATTY ACID	Control	OA 10M	OA 50M
C12:0	0.16 ± 0.11	0.22 ± 0.11	0.15 ± 0.04
C14:0	1.50 ± 0.39	1.29 ± 0.25	1.23 ± 0.34
C15:0	0.41 ± 0.05	0.43 ± 0.05	0.41 ± 0.05
C16:0	20.68 ± 2.36	17.89 ± 0.88	17.05 ± 2.90
C17:0	1.17 ± 0.13	1.01 ± 0.09	0.97 ± 0.16
C18:0	26.83 ± 5.30	20.59 ± 2.27	18.50 ± 3.76
C20:0	0.42 ± 0.05	0.35 ± 0.05	0.31 ± 0.08
C23:0	0.05 ± 0.02	0.06 ± 0.02	0.06 ± 0.02
C24:0	0.72 ± 0.26	1.03 ± 0.34	1.07 ± 0.37
ΣSFA	**51.94 ± 8.68**	**42.87 ± 2.93**	**39.75 ± 7.40**
C14:1	0.04 ± 0.03	0.02 ± 0.02	0.02 ± 0.02
C16:1	1.79 ± 0.33	1.76 ± 0.48	1.68 ± 0.30
C17:1	0.46 ± 0.31	0.58 ± 0.36	0.60 ± 0.34
C18:1n-9t	0.23 ± 0.10	0.16 ± 0.11	0.09 ± 0.03
C18:1n-9c	18.23 ± 2.98	24.47 ± 5.42	29.53 ± 9.06[b]
C18:1n-7	5.24 ±0.73	5.40 ±1.02	5.35 ± 0.57
C20:1n-9	0.34 ± 0.08	0.42 ±0.12	0.49 ± 0.15
C22:1n-9	0.35 ± 0.03	0.50 ± 0.22	0.43 ± 0.37
C24:1	0.39 ± 0.21	0.38 ± 0.24	0.64 ± 0.17
ΣMUFA	**27.07 ± 4.79**	**33.69 ± 4.09**	**38.83 ± 8.82**
C18:2n-6t	0.23 ± 0.04	0.22 ± 0.07	0.21 ± 0.07
C18:2n-6c	4.25 ±1.10	5.29 ± 1.76	5.17 ± 1.13
C18:3n-6	0.08 ± 0.08	0.15 ± 0.13	0.15 ± 0.13
C20:3n-6	0.65 ± 0.08	0.81 ± 0.10	0.77 ± 0.18
C20:4n-6	9.40 ± 3.61	9.30 ± 3.46	8.29 ± 2.19
ΣN-6PUFA	**14.62 ± 5.10**	**15.76 ±5.00**	**14.58 ± 3.01**
C18:3n-3	0.19 ± 0.04	0.18 ± 0.06	0.18 ± 0.04
C20:3n-3	0.49 ± 0.10	0.47 ± 0.12	0.45 ± 0.06
C20:5n-3	0.94 ± 0.34	1.15 ± 0.60	0.85 ± 0.25
C22:5n-3	1.82 ± 0.45	2.47 ± 0.96	2.00 ± 1.01
C22:6n-3	2.68 ± 0.47	3.16 ±1.59	3.11 ± 0.74
ΣN-3PUFA	**6.11 ± 1.40**	**7.43 ± 1.94**	**6.59 ± 1.93**
C20:2	0.19 ± 0.04	0.21 ± 0.05	0.21 ± 0.04
C22:2	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.02

Appendix.3.5. Fatty acid profile of 46BR.1Ncell before (control) and before treatment with EPA 10µM and 50µM. Results are expressed as % weight of total fatty acid (mean ± SD) of n=3 independent experiments. (a) comparing control to EPA10µM group and (b) comparing control to EPA 50µM group. [a,b]=p≤0.05, [aa,bb]=p≤0.001,[aaa,bbb]=p≤0.001.

FATTY ACID	Control	EPA 10M	EPA 50M
C12:0	0.16 ± 0.11	0.14 ± 0.07	0.14 ± 0.07
C14:0	1.50 ± 0.39	1.15 ± 0.26	1.20 ± 0.20
C15:0	0.41 ± 0.05	0.38 ± 0.03	0.37 ± 0.03
C16:0	20.68 ± 2.36	18.37 ± 0.94	17.37 ± 0.91
C17:0	1.17 ± 0.13	1.07 ± 0.14	0.98 ± 0.11
C18:0	26.83 ± 5.30	21.33 ± 1.49	19.57 ± 1.04
C20:0	0.42 ± 0.05	0.37 ± 0.05	0.34 ± 0.08
C23:0	0.05 ± 0.02	0.07 ± 0.05	0.03 ± 0.02
C24:0	0.72 ± 0.26	0.86 ± 0.27	0.68 ± 0.06
ΣSFA	**51.94 ± 8.68**	**43.73 ± 3.31**	**40.68 ± 2.52**
C14:1	0.04 ± 0.03	0.04 ± 0.01	0.03 ± 0.01
C16:1	1.79 ± 0.33	2.00 ± 0.13	1.98 ± 0.14
C17:1	0.46 ± 0.31	0.42 ± 0.28	0.41 ± 0.31
C18:1n-9t	0.23 ± 0.10	0.20 ± 0.08	0.14 ± 0.03
C18:1n-9c	18.23 ± 2.98	19.45 ± 1.86	18.01 ± 0.95
C18:1n-7	5.24 ±0.73	5.47 ± 0.26	4.85 ± 0.09
C20:1n-9	0.34 ± 0.08	0.31 ± 0.08	0.24 ± 0.03
C22:1n-9	0.35 ± 0.03	0.43 ± 0.06	0.52 ± 0.21
C24:1	0.39 ± 0.21	0.50 ± 0.05	0.43 ± 0.25
ΣMUFA	**27.07 ± 4.79**	**28.83 ± 2.81**	**26.62 ± 2.01**
C18:2n-6t	0.23 ± 0.04	0.24 ± 0.06	0.20 ± 0.03
C18:2n-6c	4.25 ±1.10	5.73 ± 0.93	5.27 ± 1.09
C18:3n-6	0.08 ± 0.08	0.07 ± 0.05	0.07 ± 0.09
C20:3n-6	0.65 ± 0.08	0.78 ± 0.19	0.74 ± 0.12
C20:4n-6	9.40 ± 3.61	9.88 ± 4.26	8.38 ± 3.08
ΣN-6PUFA	**14.62 ± 5.10**	**16.70 ± 5.49**	**14.67 ± 4.40**
C18:3n-3	0.19 ± 0.04	0.24 ± 0.02	0.31 ± 0.03[b]
C20:3n-3	0.49 ± 0.10	0.57 ± 0.09	0.45 ± 0.11
C20:5n-3	0.94 ± 0.34	2.67 ± 0.19[aaa]	6.57 ± 0.42[bbb]
C22:5n-3	1.82 ± 0.45	4.03 ± 0.75[a]	7.34 ± 1.49[bbb]
C22:6n-3	2.68 ± 0.47	2.96 ± 0.59	3.20 ± 0.51
ΣN-3PUFA	**6.11 ± 1.40**	**10.47 ± 1.64**	**17.86 ± 2.57[bb]**
C20:2	0.19 ± 0.04	0.18 ± 0.02	0.14 ± 0.01
C22:2	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01
ΣN-9PUFA	**0.23 ± 0.05**	**0.22 ± 0.04**	**0.17 ± 0.02**

Appendix.3.6. Fatty acid profile of 46BR.1Ncells before (control) and post treatment with DHA 10µM and 50µM. Results are expressed as % weight of total fatty acid (mean ± SD) of n=3 independent experiments. (a) comparing control to DHA10µM group and (b) comparing control to DHA 50µM group. [a,b]=p≤0.05, [aa,bb]=p≤0.001,[aaa,bbb]=p≤0.001,

FATTY ACID	Control	DHA 10M	DHA 50M
C12:0	0.16 ± 0.11	0.13 ± 0.05	0.12 ± 0.03
C14:0	1.50 ± 0.39	1.31 ± 0.17	1.29 ± 0.23
C15:0	0.41 ± 0.05	0.43 ± 0.07	0.43 ± 0.11
C16:0	20.68 ± 2.36	18.27 ± 1.37	17.10 ± 1.11
C17:0	1.17 ± 0.13	1.08 ± 0.09	0.75 ± 0.46
C18:0	26.83 ± 5.30	21.14 ± 1.60	19.91 ± 1.00
C20:0	0.42 ± 0.05	0.37 ± 0.05	0.31 ± 0.07
C23:0	0.05 ± 0.02	0.06 ± 0.03	0.04 ± 0.02
C24:0	0.72 ± 0.26	1.09 ± 0.40	0.93 ± 0.49
ΣSFA	**51.94 ± 8.68**	**43.88 ± 3.36**	**40.88 ± 2.92**
C14:1	0.04 ± 0.03	0.02 ± 0.03	0.02 ± 0.02
C16:1	1.79 ± 0.33	2.03 ± 0.24	2.01 ± 0.10
C17:1	0.46 ± 0.31	0.60 ± 0.43	0.54 ± 0.39
C18:1n-9t	0.23 ± 0.10	0.14 ± 0.07	0.12 ± 0.05
C18:1n-9c	18.23 ± 2.98	19.53 ± 1.61	17.82 ± 1.52
C18:1n-7	5.24 ±0.73	5.65 ± 0.39	5.12 ± 0.56
C20:1n-9	0.34 ± 0.08	0.42 ± 0.25	0.27 ± 0.07
C22:1n-9	0.35 ± 0.03	0.34 ± 0.09	0.52 ± 0.10
C24:1	0.39 ± 0.21	0.31 ± 0.19	0.30 ± 0.12
ΣMUFA	**27.07 ± 4.79**	**29.05 ± 2.31**	**26.72 ± 2.22**
C18:2n-6t	0.23 ± 0.04	0.22 ± 0.04	0.21 ± 0.03
C18:2n-6c	4.25 ±1.10	5.75 ± 1.30	6.06 ± 1.38
C18:3n-6	0.08 ± 0.08	0.22 ± 0.24	0.15 ± 0.11
C20:3n-6	0.65 ± 0.08	0.99 ± 0.12[a]	0.98 ± 0.12[a]
C20:4n-6	9.40 ± 3.61	9.49 ± 2.79	8.27 ± 2.50
ΣN-6PUFA	**14.62 ± 5.10**	**16.68 ± 3.65**	**15.66 ± 3.42**
C18:3n-3	0.19 ± 0.04	0.21 ± 0.05	0.26 ± 0.05
C20:3n-3	0.49 ± 0.10	0.48 ± 0.11	0.40 ± 0.08
C20:5n-3	0.94 ± 0.34	1.32 ± 0.19	1.38 ± 0.36
C22:5n-3	1.82 ± 0.45	2.27 ± 0.96	2.08 ± 0.64
C22:6n-3	2.68 ± 0.47	5.88 ± 2.51	15.06 ± 7.29[bbb]
ΣN-3PUFA	**6.11 ± 1.40**	**10.16 ± 3.19**	**19.17 ± 7.29[bb]**
C20:2	0.19 ± 0.04	0.21 ± 0.04	0.20 ± 0.07
C22:2	0.04 ± 0.01	0.04 ± 0.02	0.04 ± 0.02